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CANADIAN THESES ON MICROFICHE / THÈSES CANADIENNES SUR MICROFICHE

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TITLE OF THESIS/TITRE DE LA THÈSE Assessment of Gentamicin Sensitivity of Pseudomonas aeruginosa in an animal model and by accumulation studies

UNIVERSITY/UNIVERSITÉ University of Alberta

DEGREE FOR WHICH THESIS WAS PRESENTED/ GRADE POUR LEQUEL CETTE THÈSE FUT PRÉSENTÉE M Sc

YEAR THIS DEGREE CONFERRED/ANNÉE D'OBTENTION DE CE GRADE 1977

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ASSESSMENT OF GENTAMICIN SENSITIVITY OF *PSEUDOMONAS AERUGINOSA* IN AN ANIMAL MODEL AND BY ACCUMULATION STUDIES

by

© THALIA I. NICAS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

IN

MEDICAL BACTERIOLOGY

DEPARTMENT OF MEDICAL BACTERIOLOGY

EDMONTON, ALBERTA

FALL, 1977

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled ASSESSMENT OF GENTAMICIN SENSITIVITY OF *PSEUDOMONAS AERUGINOSA* IN AN ANIMAL MODEL AND BY ACCUMULATION STUDIES submitted by Thalia I. Nicas in partial fulfilment of the requirements for the degree of Master of Science in Medical Bacteriology.

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ABSTRACT

In this study we have attempted to compare in vivo and in vitro gentamicin susceptibility for seven well characterized strains of *Pseudomonas aeruginosa*. The strains included a hypersensitive strain, typical sensitive strains and strains with enzymatic and with permeability mediated resistance. Four isogenic strains differing in the R factor carried were included in an attempt to minimize variations in virulence.

In examining 11 isogenic strains with R factors, it was found that in most cases R factors did not affect virulence. Four strains showed a decrease in virulence relative to the parent strain and a single strain showed enhanced virulence.

In vivo tests were done using a mouse protection test with an inoculum of 50 LD_{50}. Both normal mice and mice made neutropenic with cyclophosphamide were used. It was found that serum levels of gentamicin required for protection were much higher than the MIC. Only the hypersensitive strain could be treated at antibiotic levels considered safe in humans. Other strains considered sensitive or moderately resistant in vitro required much higher levels of antibiotic, and no protection was possible with strains classed as resistant. Protective levels in neutropenic animals were slightly lower than those in normal mice. A distinct inoculum effect was found in the mouse tests, with a 10-fold increase in inoculum producing a four-fold increase in the amount of gentamicin required.

MIC were found to be in best agreement with in vivo results when they were done in commercial media with relatively high divalent cation concentration. When MIC were done in minimal media with varying

magnesium and calcium concentration, it was found that media with the highest cation concentration gave highest MIC.

The relationship between gentamicin uptake and sensitivity was used to devise a rapid alternative method for sensitivity testing. It was found that at one µg/ml gentamicin resistant strains of *Pseudomonas aeruginosa* failed to take up gentamicin while sensitive strains showed appreciable accumulation.

ACKNOWLEDGEMENTS

I wish to thank Dr. L.E. Bryan for his able supervision throughout the course of this investigation. I would also like to thank Dr. F.L. Jackson for his guidance during Dr. Bryan's absence.

I extend my thanks to all members of the Department of Medical Bacteriology for their assistance throughout the course of this program. I am particularly grateful to Diane Campbell and Jill Jackson for providing materials, to Dorothy Noldner for her help with animal care and especially to Debbie Berg for her careful typing of this thesis.

TABLE OF CONTENTS

	Page
ABSTRACT	iv
ACKNOWLEDGEMENTS	vi
LIST OF ABBREVIATIONS	ix
LIST OF TABLES	x
LIST OF FIGURES	xii
INTRODUCTION AND LITERATURE REVIEW	1
MATERIALS AND METHODS	13
Bacteria	13
Media	13
Antibiotics	14
Mice	15
Reagents	15
Disc Sensitivity Tests	15
Minimal Inhibitory Concentration Determinations	15
Determination of Serum Levels of Gentamicin	16
Testing of Acute Toxicity of Gentamicin	18
Virulence Testing	18
Induction of Neutropenia	19
Mouse Protection Tests	19
Characterization of Strains After Passage	20
Minimal Bactericidal Gentamicin Levels in Mouse and Human	20
Purification of Triatiated Gentamicin	21
Gentamicin Accumulation	22

Page

MATERIALS AND METHODS (continued)

Dry Weight Determination 23

RESULTS 24

1. Comparison of Isogenic R^+ and R^- Strains 24

Resistance Markers and P Groups of R factors 24

Virulence 25

Retention of the R factor after Passage through Mice . . 29

2. Mouse Protection Tests 29

Properties of Strains used for Protection Tests 29

Virulence Determinations 32

Induction of Neutropenia 34

Serum Levels of Gentamicin 38

Protection Tests 45

3. Minimal Inhibitory Concentrations of Gentamicin 55

4. Uptake Studies 59

DISCUSSION 67

LITERATURE CITED 80

LIST OF ABBREVIATIONS

Acetyl CoA	acetyl coenzyme A
A_{600}	absorbance at 600 nanometers
BHI	brain-heart infusion broth
cfu	colony forming units
dpm	disintigrations per minute
ip	intraperitoneal
kan^r	kanamycin resistant
LD_{50}	median lethal dose
met^-	auxotrophic for methionine
MBC	minimal bactericidal concentration(s)
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth
MIC	minimal inhibition concentration(s)
NB	nutrient broth
PD_{50}	median protective dose
rif^r	rifampicin resistant
sc	subcutaneous(ly)
R factor	resistance transfer factor
R+, R-	with R factor; without R factor

LIST OF TABLES

Table		Page
1.	Resistance markers and P groups of R factors	25
2.	Virulence of *P. aeruginosa* 280 R^- and 280 R^+ for normal mice	26
3.	LD_{50} of 280 R^- and 280 R^+ strains	28
4.	Virulence of *P. aeruginosa* ML4262 R^- and ML4262 R^+ for mice	30
5.	Properties of strains used in mouse protection tests	31
6.	Virulence of non-isogenic *P. aeruginosa* strains for normal mice	33
7.	Virulence of *P. aeruginosa* for neutropenic mice	36
8.	LD_{50} of *P. aeruginosa* for normal and neutropenic mice	37
9.	Peak serum levels of gentamicin in mice measured by broth dilution and radio enzymatic methods after a single sc dose	39
10.	Rate of clearance of gentamicin after single sc dose	43
11.	Gentamicin levels after repeated doses	44
12.	Comparison of single versus multiple dose schedule for protection of mice against *P. aeruginosa* infection	46
13.	Protection by gentamicin of normal mice against *P. aeruginosa*	47
14.	Protection by gentamicin of neutropenic mice against *P. aeruginosa* infection	48
15.	Gentamicin PD_{50} values for *P. aeruginosa* infection in normal mice	50
16.	Comparison of MIC and serum levels of gentamicin required for PD_{50} in normal and neutropenic mice	51
17.	Comparison of MIC of *P. aeruginosa* before and after passage through mice	53
18.	Effect of size of bacterial inoculum on gentamicin PD_{50} values for neutropenic mice	54
19.	Effect of bacterial inoculum on MIC	56

Table		Page
20.	Minimal inhibitory concentration of gentamicin in commercial media	57
21.	Minimal inhibitory concentration of gentamicin in defined media	58
22.	Uptake by *P. aeruginosa* of gentamicin	60
23.	Gentamicin uptake by sensitive and resistant strains of *P. aeruginosa*	64

LIST OF FIGURES

Figure		Page
1.	Effect of cyclophosphamide on mouse white blood cell count	35
2.	Peak serum levels of gentamicin	40
3.	Clearance of gentamicin	42
4.	Energy-dependent uptake of gentamicin by *P. aeruginosa* at 0.5 µg gentamicin/ml	61
5.	Energy dependent uptake of gentamicin by *P. aeruginosa* at 1.0 µg gentamicin/ml	62
6.	Energy dependent uptake of gentamicin by sensitive and resistant bacteria	66

INTRODUCTION AND LITERATURE REVIEW

In vitro sensitivity tests are designed to predict the effectivness of antibiotics in infected patients. However, it is axiomatic that in vitro susceptibility will not necessarily predict efficacy in man. An elegant proof of this concept has been provided by Dawkins and Hornick (1967) in their studies of experimental typhoid in man. Cephaloridine, polymixin and several other antibiotics were highly active against test strains of *Salmonella typhosa* in vitro, but only chloramphenicol and ampicillin were effective in man. Ideally, conclusive demonstration of efficacy of an antibiotic requires in vitro studies, chemotherapeutic trials in experimental infections in animals, and controlled chemotherapeutic trials in man. Antibiotics used for the treatment of pseudomonas infection have not been subject to systematic analysis in this manner (Davis *et al.*, 1971).

Pseudomonas aeruginosa is among the leading causes of hospital acquired infection. While harmless to a healthy, uninjured host, it may pose a severe threat to patients with impaired host defenses. *P. aeruginosa* sepsis is a leading cause of death among cancer patients. Steroids, cytotoxic agents and other immunosuppressants increase susceptibility. Patients with severe burns are also a highly susceptible group. *P. aeruginosa* infection commonly occurs in patients with cystic fibrosis and other chronic pulmonary disease. It has also been reported in neonates and in patients requiring instrumentation which can introduce bacteria to susceptible tissues.

Under the conditions of poor host resistance in which opportunistic pseudomonas infections occur, antibiotics are poorly supported by the

humoral and cellular host defences which complement therapeutic action when defences are normal. In patients with severe underlying illness and impaired host defences, there is little room for error or variability in laboratory estimation of susceptibility.

Only a small number of antibiotics show significant in vitro activity against *P. aeruginosa*. These are the polymixins, carbenicillin and its newer analogues, and gentamicin and the more recent aminoglycosides. Gentamicin, either alone or in combination with carbenicillin, is currently considered one of the most useful antibiotics against *P. aeruginosa*.

The aminoglycoside antibiotics, which include gentamicin are a group of substances which contain amino sugar and amino cyclitol moieties (except for spectinomycin which lacks an amino sugar). There are two major subclasses: those which contain streptidine as their cyclitol components, and those which contain 2-deoxystreptamine. Gentamicin is a member of this second group, as are tobramycin and the new semisynthetic aminoglycosides, amikacin and netilmicin. These agents have a number of effects on the bacterial cell, but their primary action is considered to be the inhibition of protein synthesis. It has been shown that streptomycin, the best studied aminoglycoside, binds to the 30S ribosomal subunit (Cox *et al.*, 1964; Davies, 1964), resulting in inhibition of protein synthesis and misreading of mRNA (Davies *et al.*, 1964; Modolell and Davies, 1969). Gentamicin and other 2-deoxystreptamine-containing aminoglycosides induce a higher level of misreading than streptomycin, and may act at more than one 30S ribosomal site (Davies *et al.*, 1969).

Gentamicin as used commercially is a complex of gentamicins C_1, C_{1a} and C_2 (Wagman *et al.*, 1968). It is not absorbed orally and must be

administered parenterally. It is not metabolized and is eliminated from the body by glomerular filtration (Black *et al.*, 1964). As with other aminoglycosides, maximum dose is limited by possible ototoxicity and nephrotoxicity (Wersall *et al.*, 1969; Falco *et al.*, 1969). Blood levels above 10-15 µg/ml are considered potentially toxic. Gentamicin has been used as a reference point and comparative standard in the evaluation of new aminoglycosides and, to some extent, other new antibiotics active against pseudomonas. However, many questions remain concerning the therapeutic efficacy of gentamicin in pseudomonas infection, and the relationship of in vitro and in vivo susceptibility.

Antibiotics active against *P. aeruginosa* have not been subject to systematic clinical evaluation. The potentially life-threatening nature of many pseudomonas infections largely precludes controlled human tests. Generally, antibiotics active against *P. aeruginosa* in vitro have been used in man in uncontrolled trials. Despite many years of clinical experience and empirical evaluation, the opinion persists that there is no adequate proof for the efficacy of antibiotics in serious pseudomonas sepsis (Davis *et al.*, 1971; Flick and Cluff, 1976).

Evaluation of antibiotic treatment of pseudomonas sepsis is confounded by the fact that such infections generally occur secondarily to a serious predisposing disease. The effects of primary diseases such as burns, leukemias, malignancies and immunosuppression and cystic fibrosis are not easily separated from the effects of the complicating infection. In many cases, control of the primary disease is considered more critical in the outcome of pseudomonas sepsis than is appropriate antibiotic therapy (Tapper and Armstrong, 1974; Flick and Cluff, 1976). This is especially true for granulocytopenic patients (Tapper and Arm-

strong, 1974; Bodey, 1975). Another problem is that the distinction between pseudomonas colonization and active infection has sometimes been inadequately examined. In total, these circumstances have made the results of clinical trials very difficult to interpret. Evaluation of the efficacy of any single agent is further complicated by the fact that combinations of drugs are often used in therapy of pseudomonas infection.

The effectiveness of antibiotics against pseudomonas infection is dependent to a high degree on the nature of the infection. For example urinary tract infections represent a special case of relatively easily controlled infections because of the high levels of antibiotic which may be attained in urine. Comparable antibiotic efficacy cannot be expected for other more severe or widely disseminated infections.

Endocarditis is one type of infection in which complicating effects are at minimum. In a study of pseudomonas endocarditis Reyes *et al.* (1971 and 1975) were able to show that the effectiveness of antibiotic treatment was appreciably increased when relatively high doses were used. Infections were eradicated in only 25% of patients over the period in which 2.5-5.0 mg/kg/day of gentamicin or tobramycin with 30 g/day carbenicillin was used while the same dose of carbenicillin and 8 mg/kg of gentamicin or tobramycin improved the cure rate to 58%

For gentamicin in particular, there is no overall agreement as to its efficacy in the treatment of pseudomonas infection. The literature on the treatment of pseudomonas bacteremia provides a clear example of this. Reported success rates range from very good to very poor. Stone *et al.* (1965) reported survival of 10 out of 13 severely burned patients with pseudomonas septicemia. Good results are also reported by Mueller (1967) and by Cox and Harris (1971). In contrast, Jackson and

Riff (1971) and McGowan *et al.* (1975) report respectively 71% (15 out of 20) and 64% (14 out of 22) mortality rates in their studies of pseudomonas bacteremia. Several authors maintain that in their clinical experience gentamicin is not effective in granulocytopenic hosts, whereas carbenicillin may be (Reynolds *et al.*, 1975; Bodey, 1975). Flick and Cluff (1976), in a study of 108 cases of pseudomonas bacteremia claim no decrease in mortality from pseudomonas bacteremia over a 25-year period (1951-1976), despite the introduction of gentamicin and carbenicillin.

Overall, studies of human pseudomonas infection have for the most part failed to provide a clear picture of antibiotic efficacy. Studies of antibiotic efficacy in severe, disseminated infections are the least likely to yield unambiguous results because of the many problems in interpretation, yet these are the circumstances in which the need for such information is most pressing. Animal studies designed to be comparable to this type of infection may present an alternative system of evaluation.

Animal studies have also produced equivocal results with respect to the efficacy of gentamicin. Successful treatment of pseudomonas infection in animals is reported, but often the dosages required are high on a weight basis, and the correlation between in vitro and in vivo susceptibility is poor.

Among the first reports of gentamicin treatment of pseudomonas infection is that of Summerlin and Artz (1966). Rats with pseudomonas infected full thickness skin wounds were given 20 mg/kg daily for 21 days. Mortality in untreated rats was 20 out of 30, while 14 of 15 rats receiving immediate treatment and 11 of 15 rats in which treatment was

delayed survived the infection. Rosenthal (1968) used a burned mouse model to study the effects of antibiotics in pseudomonas infection. In his study 15 mg/kg of gentamicin protected 40% of animals with systemic infections. Smith (1971) was able to protect 60-100% of normal mice from lethal ip infection by four pseudomonas strains using a dose of about 20 mg/kg. One of the more favorable reports is that of Saslaw *et al.* (1972). Using a small number of monkeys made susceptible by vincristine treatment and a single strain of *P. aeruginosa*, they were able to show a decrease in mortality with 2.5 mg/kg of gentamicin daily for ten days. This dose is much lower than that used successfully in other animal studies.

Lumish and Norden (1977) studied infection in rats made neutropenic with cyclophosphamide. They were able to show reduced mortality from pseudomonas infection using a single dose of 10 mg/kg, three times a day, which they found gave a peak serum level of 9.8 µg/ml. In a similar study, Scott and Robson (1976) found that a lower dose of gentamicin, 6 mg/kg three times a day was not successful in preventing the death of pseudomonas-infected neutropenic rats. Both of these studies found that combined gentamicin and carbenicillin treatment was more successful than gentamicin alone.

Weinstein *et al.* (1971) used mouse protection tests to compare protective levels of gentamicin with susceptibility measured by zone size in Kirby-Bauer testing and MIC for 82 *P. aeruginosa* strains. He found a low correlation between in vivo and in vitro antibacterial activity. Waitz *et al.* (1972) examined six strains of *P. aeruginosa* sensitive to gentamicin by in vitro tests and found that in the mouse protection test the range of PD_{50} values they gave was 0.5 to 19 mg/kg,

with a mean of 7.8 mg/kg. In a similar study Heifetz *et al.* (1974) examined eight strains all sensitive according to the Kirby-Bauer test, and found PD_{50} values of 7.2 to 16.1 mg/kg. In this study the peak serum levels of gentamicin at the PD_{50} values were at least eight times higher than the MIC in six out of the eight strains. Davis (1975) found even larger differences in the in vivo response among strains considered susceptible in vitro. Among six strains studied in the mouse protection test, PD_{50} values ranged from 1.6 to 62. All of these six strains were sensitive in vitro, with MIC less than six µg/ml, but only two could be considered susceptible in vivo. PD_{50} from mouse protection tests done in different laboratories are not strictly comparable because of differences in inoculum size and dose schedule. Nonetheless, dissociation between in vivo and in vitro antibiotic activity is generally reported.

In vitro testing of the susceptibility of *P. aeruginosa* to gentamicin also presents problems. The apparent susceptibility of *P. aeruginosa* to gentamicin and other aminoglycosides is influenced to a uniquely high degree by the method of testing used. pH may have a major influence on apparent susceptibility, but this variable is generally well controlled. The effect of calcium and magnesium content on aminoglycoside resistance in *P. aeruginosa* has been extensively documented (Donovich, 1948; Waitz and Weinstein, 1969; Traub, 1970; Garrod and Waterworth, 1970; Washington *et al.*, 1970; Madeiros *et al.*, 1971; Davis and Iannetta, 1972; Zimellis and Jackson, 1973; Reller, 1974). Increase in the calcium and magnesium content of the media result in higher resistance. This is not exclusively a function of ionic strength, but is specific to divalent cation (Madeiros *et al.*, 1971; Beggs *et al.*, 1974). Differences in MIC of gentamicin of four to 32 fold may occur among different commercial

media (Ganrod and Waterworth, 1970). Thirty-two fold differences have been found among MIC done in different batches of Mueller Hinton media with varying calcium and magnesium levels (Reller *et al.*, 1974). Resistance of *E. coli* and other enterobacteriacae to aminoglycosides do not show comparable dependence on divalent cation. The protective effect of calcium and magnesium on *P. aeruginosa* is usually attributed to stabilization of the cell envelope by divalent cation, with the resultant effect of decreased permeability to aminoglycoside (Zimelis and Jackson, 1973; Gilbert *et al.*, 1971). A protective effect of divalent cation for polymixin antibiotics has also been shown for *P. aeruginosa* (Newton, 1953; Newton, 1959; Davis *et al.*, 1971b). No parallel effect can be demonstrated for carbenicillin (Davis and Iannetta, 1972).

The need for standard levels of magnesium and calcium is illustrated by the finding of Gilbert *et al.* (1971) that among 18 typical hospital isolates the proportion that could be considered gentamicin resistant (MIC of eight µg/ml or more) varied from none to 72 per cent. Variation in media makes evaluation and comparison of reported in vitro susceptibility difficult. Use of media with low divalent cation content may result in underestimation of susceptibility and consequently inappropriate therapy. The high fatality rates often reported for pseudomonas sepsis and the high serum levels required for protection in most animal studies are suggestive of this situation.

MIC done in the presence of serum are usually reported as higher than those done in broth alone. However, Davis and Iannetta (1972) have shown that the increase in resistance brought about by the addition of serum can be duplicated by the addition of calcium equivalent to serum levels.

Standardization of media using serum concentrations of magnesium and ionized calcium has been suggested, (Reller *et al.*, 1974; Bryan *et al.*, 1976), but is not widely used. Bryan and coworkers (Bryan and Van Den Elzen, 1977; Bryan *et al.*, 1976) report that when cation concentrations are adjusted to physiological levels, the MIC for gentamicin for most strains of *P. aeruginosa* will be in the range of two to eight µg/ml.

Emergence of gentamicin resistance in *P. aeruginosa* was first noted in burn units, where high levels of gentamicin were used in topical therapy (Mueller, 1967; Shulman *et al.*, 1971). The first reported mechanism of resistance to gentamicin was inactivation by modification of the antibiotic by R factor specified enzymes (Witchitz and Chabbert, 1971 and 1972; Brzesinska *et al.*, 1972). At least seven R factor coded enzymes capable of modifying gentamicin are now known (Haas and Dowding, 1975; Price *et al.*, 1976; Dowding, 1977). At present five enzymatic activities capable of inactivation of gentamicin have been demonstrated in various *P. aeruginosa* strains. These comprise two enzymes carrying out 3-N-acetylation, (Brzesinska *et al.*, 1972; Biddlecome *et al.*, 1976), two with 6'-N-acetylation activity (Kawabe *et al.*, 1975; Haas *et al.*, 1976) and an enzyme carrying out adenylylation at the 2" position (Kabins *et al.*, 1974). Adenylylation appears to be the most commonly occuring mechanism for R factor mediated gentamicin resistance.

In the case of streptomycin and other streptidine containing aminoglycosides, resistance may occur as the result of mutation causing modification of the 30S ribosomal subunit so that it is no longer capable of binding the antibiotic. Ribosomal resistance to gentamicin and other 2-deoxystreptamine aminoglycosides has never been shown. An early report of ribosomal resistance to gentamicin (Tanaka, 1970) was later shown to

be due to an acetylating enzyme (Brzesinska, 1972).

In a survey of *P. aeruginosa* isolates at University of Alberta, Bryan, Haraphongse and Shahrabadi (1974) found that gentamicin resistance could not be attributed to enzymatic mechanism in a high proportion of strains. The majority of resistant strains showed no aminoglycoside acetylating, adenylylating or phosphorylating activity, nor did they have resistant ribosomes. Some of these strains had plasmid DNA, but they were unable to transfer resistance. In subsequent work, they were able to demonstrate that enzyme-negative resistant strains differed from sensitive strains in their ability to accumulate aminoglycoside (Bryan, Haraphongse and Shahrabadi, 1974; Bryan, Van Den Elzen and Shahrabadi, 1975).

Permeability to aminoglycoside appears to be the critical variable in determining the sensitivity of a strain to gentamicin. Bryan and Van Den Elzen (1975 and 1975b) have described cellular entry of aminoglycosides in detail. The process occurs in three steps. The first of these appears to be an ionic binding to the cell wall without entry into the cell or loss of viability. The remaining phases may be eliminated by inhibitors of electron transport or uncouplers and are thus termed energy dependent phases I and II (EDP-I and -II). The two phases are not readily resoluble in the case of gentamicin and detailed kinetic studies have been done with streptomycin. The first phase EDP-I is rate limiting. In the absence of sensitive ribosomes, no further uptake occurs and the cells remain viable. EDP-II occurs only in the presence of sensitive ribosomes, and is characterized by much more rapid transport of aminoglycoside. The onset of this phase is associated with inhibition of protein synthesis and loss of cell viability.

By studying the accumulation of [^{3}H] gentamicin Bryan and coworkers were able to demonstrate that at low concentrations of gentamicin, sensitive strains accumulated gentamicin and resistant strains did not. The concentration at which energy dependent uptake begins corresponds to the resistance levels of the strain in the media used for uptake. Thus the resistance of a strain depends on a threshold level at which active gentamicin accumulation begins. Highly resistant strains could remain in relatively high gentamicin concentrations without antibiotic accumulating beyond the initial surface binding. Ramirez-Ronda and coworkers (1975) have confirmed the relationship between accumulation of gentamicin and cell death. They were also able to show that accumulation increased at reduced calcium and magnesium levels, as would be predicted by the increase in sensitivity seen under these conditions.

The relationship between uptake and resistance appears to be a factor in enzymatically mediated resistance as well. Accumulation of gentamicin is reduced in strains acquiring an R factor which directs enzymatic modification (Bryan and Van Den Elzen, 1975 and 1975b).

While *P. aeruginosa* resistant to gentamicin by enzymatic mechanisms usually have MIC of 100 µg/ml or higher, permeability resistance covers a very broad range from very high level resistance, to low resistance, forming a continuum with sensitive strains. Permeability resistance also shows a high degree of cross resistance among the aminoglycosides. Bryan, Haraphongse and Shahrabadi (1974) found that of three gentamicin resistant *P. aeruginosa* isolates examined, all were cross resistant to seven other aminoglycosides tested.

In this study we have attempted to compare in vivo and in vitro gentamicin susceptibility for seven well characterized strains of

P. aeruginosa. The strains used include a hypersensitive strain, typical sensitive strains and strains with both enzymatic and permeability mediated resistance. Four isogenic strains (a parent strain and three R factor carrying derivitives) were included in an attempt to minimize variation in virulence. In the in vitro studies we examined some effects of differences in media and divalent cation content. The method commonly called the mouse protection test was used as the principal method of in vivo evaluation. This technique allowed us to examine the effects of virulence, inoculum size and dose schedule on in vivo susceptibility. Neutropenic animals were used in addition to normal mice in order to mimic conditions in which pseudomonas infections are most likely to occur. Evaluation of protective levels of gentamicin in an animal system should have at least as much relevance for human therapy as any in vitro test. Thus we hope this study may have some practical implications.

We have attempted to use the relationship between gentamicin uptake and gentamicin susceptibility to devise an alternate method of susceptibility determination. We have used accumulation studies to develop a simple, rapid sensitivity test which may find practical application.

MATERIALS AND METHODS

Bacteria

Pseudomonas aeruginosa strains 280 and ML4262 and their R factor carrying derivatives were obtained from the culture collection of L.E. Bryan, Department of Bacteriology, University of Alberta. *P. aeruginosa* strains 1136, 10804, 3505, 8803, 13934, POW-151 and PS-130 were also obtained from this collection. Grouping of R factors with respect to P group was done under the system proposed by Bryan (1973) and Shahrabadi, Bryan and Van Den Elzen (1975).

P. aeruginosa strain PA-103 was generously provided by S.D. Davis (Medical College of Wisconsin, Milwaukee, Wisconsin).

All other strains were kindly provided by the antibiotic sensitivity section of the University of Alberta Hospital bacteriology laboratory.

Strains were maintained at 4C on tryptose agar plates with subculture every two to three weeks.

Media

The following media were obtained from the indicated sources:

Mueller Hinton Agar (MHA)	BBL
Mueller Hinton Broth (MHB)	BBL
Oxoid 'Isosensitest' Agar	Oxoid
Oxoid 'Isosensitest' Broth	Oxoid
Nutrient Broth (NB)	BBL
Tryptose Agar Base (TA)	Difco
Brain Heart Infusion Broth (BHI)	Difco

All MHA and MHB used was adjusted to calcium levels of 75 mg/l and

magnesium levels of 20 mg/l using calcium chloride and magnesium chloride. 0.05% Triton X-100 (Beckman) was added to NB to prevent clumping. A single lot of each media was used throughout the investigation.

Analysis of calcium and magnesium content of media was carried out at the University of Alberta Hospital using the atomic absorption method.

Low phosphate minimal media was prepared according to the following formula:

NH_4Cl	500 mg
NH_4NO_3	100 mg
$Na_2SO_44 \cdot H_2o$	100 mg
$MnSO_47 \cdot H_2o$	0.1 mg
$FeSO_47 \cdot H_2O$	0.05 mg
K_2HPO_4	28 mg
K_2MPO_4	12 mg
Tris(hydroxymethyl) aminomethane HCl	1211 mg
peptone	2.5 mg
Distilled water to	1000 ml

Final pH was 7.4. After autoclaving $MgSO_47 \cdot H_2O$ to 4 mg/l of magnesium and glucose to 0.2% were added.

Modifications of this media were prepared by the addition of further magnesium and calcium as sulphate salts.

Antibiotics

Gentamicin sulphate injectable (40 mg base/ml) was obtained from Schering Corporation Limited. Gentamicin was tritiated by catalytic

tritium exchange by the Radiochemical Centre, Amersham, England.

Mice

Male ICR mice weighing 25-31 g were used. Mice were obtained from the Health Sciences Breeding Unit, University of Alberta and maintained in groups of five or less in plastic cages with mouse chow and water *ad libitum*.

Reagents

All chemicals were of reagent and were obtained from commercial suppliers. Cyclophosphamide powder was obtained from Frank W. Horner Ltd., Montreal, P.Q.

Disc Sensitivity Tests

These were done by the Kirby-Bauer method, (Bauer *et al.*, 1966) on Mueller Hinton Agar.

Minimal Inhibitory Concentration Determinations

Broth dilution method. Conventional tube-dilution procedure was carried out using a volume of one ml broth in 16 x 125 mm tubes. Inocula used were prepared from broth culture grown in the same media used for the dilution series adjusted to 0.5 absorbancy units at 600 nm (A_{600}) on a Unicam SP 1800 spectophotometer (approximately 10^9 organisms per ml). Adjusted cultures were diluted by a factor of 10^3 in the same media, and 0.1 ml per tube of these dilutions added to give a final concentration of approximately 10^5 organisms per ml. Endpoints were determined visually after 24 h with the exception of those dilution series done in NB which were read after 48 h.

Agar dilution method. The Steers replicator method (Steers 1954) was carried out using as inocula broth cultures adjusted to an A_{600} of 0.5 as above. Mueller Hinton broth was used to grow inocula for Mueller Hinton agar and 'Isosensitest' broth for 'Isosensitest' agar. The endpoint was determined as spots with less than five colonies after 24 h.

Determination of Serum levels of Gentamicin

Gentamicin adminstration and serum collection. Gentamicin was diluted in sterile distilled water for administration in volumes of about 0.15 ml per mouse. This preparation was injected subcutaneously (sc) in the back or thigh. Each mouse was weighed to the nearest gram to establish correct dose.

Fifty to 60 µl of blood per mouse was collected by tail bleed, and blood from three or four mice pooled for each sample. Serum was collected by centrifugation using a Beckman 152 microfuge. For levels taken over extended periods of time, the same group of mice was used for all readings.

Gentamicin levels were first estimated by a broth dilution method then established more precisely by the radioenzymatic assay.

Broth dilution method. Serial dilutions of gentamicin standard or serum to be tested were prepared in 0.1 ml volumes of NB in 8 x 75 mm test tubes. 0.1 ml of a 10^{-4} dilution of *P. aeruginosa* 280 grown to an A_{600} of 0.5 was added to give about 10^5 bacteria per ml in a total volume of 0.2 ml. The endpoint was determined visually after 24 h. The minimal inhibitory level for standard was 0.008 to 0.016 µg/ml.

Radioenzymatic method. The method used was the modification of the method of Haas and Davies (1973) used by the University of Alberta Hospital.

The buffer was 0.3M Tris(hydroxymethyl)aminomethane HCl pH 7.8, 0.03M magnesium chloride and 0.08M β mercaptoethanol. Acetyl-1-[^{14}C] coenzyme A (49mCi/mM) obtained from New England Nuclear was diluted with cold acetyl CoA to 0.006 µCi/µl and stored in 0.5 ml aliquots at -20C. The concentration of acetyl CoA preparation used was 0.3 mg/ml. Enzyme for the assay, acetyl coenzyme A aminoglycoside 3-N acetyltransferase (AAC(3')-I) was prepared at the University of Alberta Hospital from *E. coli* JR89 by osmotic shock.

Ten ml buffer, 10 µl acetyl CoA, 20 µl standard or test serum and 20 µl enzyme preparation were mixed in microfuge tubes then incubated in a 32C water bath for 15 minutes. 20 µl of reaction mixture was then transferred to a one-cm square of phosphocellulose paper (Whatman P-81). Papers were immersed in 80C double distilled water for two minutes to stop reaction, washed three times in 500 ml volumes of double distilled water, dried at 60C and counted in a Beckman LS-250 liquid scintillation counter using toluene scintillation fluid (Beckman Omniflour).

Disintigrations per minute (dpm) of a blank containing 8 µg gentamicin per ml of serum with buffer replacing enzyme preparation in the reaction mixture were subtracted from each count to correct for background. Corrected dpm per sample were plotted against gentamicin per ml standards, and gentamicin levels of test sera read from this plot. Standards were one, three, six and eight µg gentamicin per ml serum. Test sera falling outside this range were diluted to an appropriate level with mouse serum.

As it was found that assay of the same quantity of gentamicin resulted in much lower counts per minute in mouse serum compared to human serum, pooled mouse serum was used in preparation of standards.

Testing of acute toxicity of gentamicin

Groups of 10 mice were given single or multiple doses of gentamicin prepared and administered as for determination of serum levels above. Mice were observed for deaths for six days. Control mice were given the same number of doses of normal saline. LD_{50} were determined by the Reed-Muench method (Reed and Muench, 1938).

Virulence Testing

Organisms to be used for virulence testing were grown overnight in BHI in shaking flasks at 37C then subcultured to about 50 ml volumes of BHI in 125 ml flasks and grown at 37C with shaking to an A_{600} of 0.50 (Unicam SP 1800 spectophotometer). Bacteria were then spun down at 8000 rpm for 10 minutes (Serval RC-2 centrifuge) and resuspended in BHI to the original volume or 0.1 of the original volume. Serial tenfold dilutions were prepared from the original volume suspension in BHI.

Mice were injected interperitoneally (ip) with 0.2 ml of cell suspension. Five mice were used for each dilution. Deaths were counted after 48 h. LD_{50} values were determined by the Reed-Muench method (Reed and Muench, 1938).

With each virulence test the number of colony forming units per ml was checked by plating 0.1 ml of a 10^{-6} dilution of the original volume suspension onto TA plates. Each count was done in triplicate and read after 18 hours.

Induction of Neutropenia

The method used was that of Pierson *et al.* (1977). A single ip dose of 300 mg/kg cyclophosphamide was used to induce neutropenia. Cyclophosphamide powder was dissolved in normal saline and administered ip in approximately 0.3 ml volumes. Less than one hour elapsed between reconstitution of the powder and administration. Mice were individually weighed to establish correct dose.

White blood cell counts were done at the time of the first dose and at 24 hour intervals using a haemocytometer,

No special precautions were taken in the maintenance and handling of mice receiving cyclophosphamide.

Mouse Protection Tests

Mice were injected ip with 0.2 ml of bacterial suspension containing 50 LD_{50} of *P. aeruginosa* per dose. Gentamicin was administered sc beginning one hour after infection. Five mice were used for each gentamicin level. Deaths were counted after 48 hours. Data were analyzed by the Spearman-Karber technique (Finney, 1971).

Organisms were prepared and administered as was done for the virulence tests, adjusting the final bacterial concentration to 250 LD_{50} per ml to give 50 LD_{50} per 0.2 ml inoculum. Gentamicin was prepared and administered sc as was done for determination of serum levels. Deaths were counted after 48 h. Injection of neutropenic animals was carried out 4 days after cyclophosphamide treatment.

Virulence controls were done with each protection test in accordance with the recommendation of Davis (1975). These consisted of one group of mice administered 50 LD_{50} with no antibiotic and mice ad-

ministered 0.5 LD_{50} with no antibiotic. Tests in which mice survived 50 LD_{50} or failed to survive 0.5 LD_{50} were discarded as invalid.

Characterization of Strains After Passage

Infecting organisms bearing R factors were recovered after death by ip wash and tested by Kirby-Bauer procedure for retention of R factors. Approximately 50 single colony isolates collected by streaking ip wash onto TA each of 280, 280(R151), 280(R130) were individually tested by replicate plating on MHA containing 1 µg/ml gentamicin and 16.5 µg/ml streptomycin.

In order to establish with certainty that the infecting organisms were the cause of death in neutropenic animals, heart tissue from dead animals and blood from moribund animals was cultured to TA plates and colonies appearing after 48 h incubation at 37C identified. 280 and its R factor carrying derivatives and strain 1136 were identified by their characteristic pigment and by antibiotic resistance patterns established by the Kirby-Bauer method. Strains PA-103 and 10804 were identified by pyocine typing done by the Provincial Laboratory of Public Health, Edmonton, Alberta using the method of Govan and Gillies (1967).

In order to check MIC of organisms after passage with minimal subculture, cardiac blood from moribund animals (containing approximately 10^6 organisms per ml) was diluted 1:10 in MHB, then grown two to three hours to an A_{600} of 0.50. These cultures were used as inoculum for MIC performed in MHB by the method described above.

Minimal Bactericidal Gentamicin Levels in Mouse and Human

Pooled human serum provided by the Provincial Laboratory of Public Health, Edmonton, and pooled serum of ICR mice were used. Serum

was stored at -20C before use.

Serial dilutions of gentamicin in serum and in 50% serum:50% MHB were prepared in 0.1 ml volumes in 8 x 75 mm tubes. To this was added an inoculum of 10 µl of a 10^{-3} dilution of a MHB culture adjusted to A_{600} of 0.5 to give a concentration of about 10^5 bacteria per ml. After 18 h incubation at 37C material from each tube was streaked on MHA and incubated a further 18 h. Less than two colonies per streak was considered bactericidal level.

Purification of Tritiated Gentamicin

[^{3}H] gentamicin was tritiated by the Radiochemical Centre, Amersham, England. The product of the catalytic exchange, crude [^{3}H] gentamicin, was purified by the method described by Bryan and Van Den Elzen (1975) and by Haraphongse (1974). Initial purification was done as described by Mahon *et al.* using Sephadex G-10 filtration. Two to three grams of Sephadex G-100 (Pharmacia, Uppsala, Sweden) was suspended in 0.02% (w/v) aqueous NaCl, packed in a 0.9 x 20 cm columns and washed with several volumes of .02% NaCl. Crude [^{3}H] gentamicin as supplied by Amersham Searle was applied to the column and eluted with 0.02% NaCl. Fractions were collected and assayed for antimicrobial activity using a disc diffusion technique. 5 µl of the fraction was put onto filter paper discs subsequently plated on a lawn of *P. aeruginosa* 280 on MHA. Plates were read after 18 h at 37C. Fractions showing antimicrobial activity were pooled, evaporated to a total volume of 0.2 ml, and further purified by the method of Wagman *et al.* (1968), using descending chromatography on Whatman No. 1 paper. The solvent was methanol-chloroform-17% ammonium hydroxide (1:2:1), with the lower phase as stationary solvent and upper phase as mobile solvent. Unlabelled gentamicin was

run on every chromatograph, and gentamicin was located by spraying the cold run with ninhydrin. Spots containing gentamicin C_1, C_{1a} and C_2 were cut out, and the gentamicin eluted in water at 20C. These preparations were filtered to remove paper particles using membrane filters with a pore size of 0.2 μ. Purified [^{3}H] gentamicin was stored at 4C and used within 30 days. Each batch was assayed for gentamicin content by the radioenzymatic assay described above. The method was modified by preparation of standards in water rather than serum. Final concentrations were 10-20 ng/ml, with specific activity of 600-700 dpm/ng. The preparation was used in uptake studies without dilution with cold gentamcin.

Gentamicin Accumulation

The method used was that described by Bryan and Van Den Elzen (1975 and 1976). Assays were done with bacteria growing at 37C with shaking in NB containing 0.05% Triton X-100 to mid log growth phase (A_{600} of 0.35-0.55 on Unicam SP 1800 spectophotometer). For kinetic assays, a total volume of 10 ml in a 125 ml flask was used for the assay. One ml of appropriately diluted [^{3}H]-gentamicin was added at the start of the assay. Samples (1.2 ml) were removed and filtered through 0.4 μ membrane filters pretreated by soaking in a 200 μg/ml solution of gentamicin. A_{600} readings of each sample were taken before filtering. Filters were washed with 20 ml 3% (w/v) saline, dried at 60C and counted in a Beckman model LS 250 liquid scintillation counter using toluene scintillation fluid (Beckman Omniflour).

In experiments using KCN treated controls, KCN was added to a final concentration of 0.1mM to growing cultures five minutes before the

addition of gentamicin.

In experiments in which a small number of samples were taken, a total volume of three to five ml in 20 ml bottles was used.

Dry Weight Determination

Bacteria were grown in Nutrient broth (with .02% Triton X-100) to an A_{600} of approximately 0.5. Ten samples of ten ml each of culture or media alone were filtered through preweighed membrane filters with 0.45 μ pore size. Each filter was washed with 10 ml normal saline. Filters were dried to constant weight, and the mean weight of the filters receiving media subtracted from that of those receiving media with bacteria to give an average dry weight per 10 ml of cultures. Three strains, 280, 1136 and 13934 were used.

The weight expressed as a function of the A_{600} value was 0.30 mg/A_{600} unit. There was little variation among strains. This figure was used in the calculation of uptake of [^{3}H] gentamicin in ng gentamicin per mg dry weight of bacteria.

RESULTS

1. Comparison of Isogenic R^+ and R^- Strains

Resistance Markers and P Groups of R factors

These properties are shown in Table 1. Derivatives of the hypersensitive strain *Pseudomonas aeruginosa* 280 (280 rif^r, 280 rif^r met^- or 280 kan^r) were the recipients harboring the R factors. Resistance markers were checked by the disc diffusion method. Classification according to P group was done as recommended by Shahrabadi *et al.* (1975).

R factors from five compatability groups were included. Four representatives of the most commonly occuring group, P2 were used.

Virulence

Representative raw data from virulence tests are given in Table 2. Mice were given 0.2 ml ip of *P. aeruginosa* suspended in BHI. Cultures were grown to 0.5 A_{600} units in BHI, centrifuged to remove culture media and resuspended in BHI at the concentrations given in the table. Resuspension of bacteria in fresh culture media reduced the possibility of illness due to carryover of preformed toxins, as well as allowing bacterial concentration.

No deaths were seen in control mice given BHI alone. Most deaths occurred within 24-36 hours. Forty-eight hours was chosen as an arbitrary cutoff point as preliminary studies failed to show any further deaths after this interval. Mice examined after death showed extensive hemorrhagic necrosis as described by Bartell *et al.* (1968).

Virulence tests were repeated two to four times for strains 280 R^-, 280(R130), 280(RUA7), 280(R151) and all additional R^+ strains in

Table 1. Resistance markers and P groups of R factors

R factor	Resistance markers carried	Group
(R130)	Gm Sm Su Hg*	P2
(R442)	Gm Sm Su Hg*	P2
(R931)	Sm Tc Hg*	P2
(Rms159)	Cm Su Tc Hg*	P2
(RP4)	Cb Km Tc	P1
(R151)	Cb Gm Sm Su	P3
(R5265)	Sm Su	P4
(Rms163)	Cm Su Tc	P5
(RUA16)	Cb Gm Km Sm	unknown
(RUA7)	Cb Gm Sm Tc	unknown
(RUA35)	Gm Km Tc	unknown

Cb carbenicillin	Sm streptomycin
Cm chloramphenicol	Su sulfonamide
Gm gentamicin	Tc tetracycline
Km kanamycin	Hg mercury

*Hg resistance reported; was not tested

Table 2. Virulence of *P. aeruginosa* 280 R^- and 280 R^+ for normal mice

R factor	Deaths per 5 mouse group			
	Concentration of suspension used as inoculum			
	x10	x1	$x10^{-1}$	$x10^{-2}$
none	5	4	0	
(R130)[a]	5	4	0	
(R442)[b]	4	4	1	0
(R931)[c]	4	3	0	
(Rms159)[c]	5	4	0	
(RP4)[a]	5	4	0	
(R151)[a]	5	5	4	0
(R5265)[c]	5	2	0	
(Rms163)[c]	5	4	0	
(RUA16)[a]	5	1	0	
(RUA7)[a]	5	4	1	0
(RUA35)[a]	5	0	0	

a carried in 280 rif^r met^-

b carried in 280 rif^r

c carried in 280 kan^r

Mice were inoculated ip with 0.2 ml of bacterial suspension prepared as described in materials and methods. Deaths after 48 h are given.

which initial tests indicated virulence differing from that of the R^- strain by more than 50 per cent. LD_{50} values from repeated tests showed a satisfactory degree of reproducibility. Most strains showed variation of LD_{50} by a factor of $10^{0.2}$ or less, and no strains varied by a factor of more than $10^{0.5}$.

A comparison of LD_{50} of 280 R^- and R^+ strains is presented in Table 3. Six of the 11 strains used (280(R130), 280(R442), 280(Rms154), 280(RP4), 280(RUA7)) have LD_{50} that do not appear to be significantly different from that of the R^- strains. These strains have LD_{50} of 0.71 to 1.1 times that of the R^- strain. This variation in virulence of 0.90 to 1.4 times that of the parent strain is no more than that observed within the same strain. This group constitutes the majority of strains tested. Four strains (280(R931), 280(R5265), 280(RUA16) and 280(RUA35)) are considerably less virulent than their R^- parent strain. The LD_{50} values of these strains range from 3.8 to 10 times higher than that of 280 R^-. This is 0.09 to 0.26 times as virulent. A single R^+ strain, 280(R151), was significantly more virulent than 280 R^-. The LD_{50} of this strain was 0.17 that of 280 R^-, an increase in virulence by a factor of 5.6.

There was no correlation between the variation in the 280 recipient carrying the R factor and the degree of virulence. 280 kan^r R^+ strains were in two of the virulence classes and 280 rif^r met^- R^+ strains in all three. Thus the kan^r rif^r and met^- genotypes of the recipient are unlikely to be of any relevance with respect to virulence.

R factors from the P2 group included three that did not affect virulence and one that decreased it. The one R factor which appeared to increase virulence, (R151), was a P3 R factor.

Table 3. LD_{50} of 280 R^- and 280 R^+ strains

R factor present	LD_{50} (bacteria/inoculum)	$\frac{LD_{50}\ 280\ R^+}{LD_{50}\ 280\ R^-}$
none	4.5×10^7	1
(R130)	4.5×10^7	1.0
(R442)	4.7×10^7	1.0
(R931)	3.1×10^8	6.9
(Rms159)	3.2×10^7	0.71
(RP4)	4.0×10^7	0.89
(R151)	8.0×10^6	0.17
(R5265)	5.0×10^8	11.1
(Rms163)	3.5×10^7	0.78
(RUA16)	4.5×10^8	10.0
(RUA7)	4.0×10^7	0.89
(RUA35)	1.7×10^8	3.8

LD_{50} values were calculated by the Reed-Muench method using virulence data and plate-count measurements of bacteria per ml of undiluted suspension.

In order to determine if R factors would have the same effect on virulence in a different recipient, R^+ and R^- strains of ML4262 were tested. One R factor not affecting virulence, (R130), one decreasing virulence, (R931), and one increasing virulence, (R151), were tested in ML4262. Results are presented in Table 4. As with 280, (R130) has no effect on virulence in ML4262. The presence of (R931) in ML4262 decreased virulence as in 280, but the magnitude of the decrease was much greater. (R151), however, did not have the same effect in ML4262 as in 280. ML4262(R151) showed no increase in virulence relative to the parent strain.

Retention of the R factor after Passage through Mice

Bacteria were recovered from dead animals by ip wash. Disc diffusion testing of recovered bacteria showed the same sensitivity patterns as bacteria before passage. To further confirm retention of R factor after passage, approximately 50 single colony isolates of 280(R130), 280(R151) and 280 R^- were collected by streaking ip wash onto TA plates, and each colony individually tested for gentamicin and streptomycin resistance. All isolates collected from 280(R130)- or 280(R151)-infected mice were resistant to both antibiotics, indicating a very high degree of R factor retention. No 280 R^- isolates were resistant.

2. Mouse Protection Tests

Properties of Strains used for Protection Tests

Strains chosen for mouse protection tests are shown in Table 5. These strains represent a very broad range of gentamicin sensitivity,

Table 4. Virulence of *P. aeruginosa* ML4262 R^- and ML4262 R^+ for mice

Strain	Deaths per 5 mouse group				LD_{50}
	Concentration of suspension used as inoculum				(bacteria/inoculum)
	x10	x1	$x10^{-1}$	$x10^{-2}$	
ML4262	4	3	1	0	1.4×10^8
ML4262(R130)	5	3	0	0	1.4×10^8
ML4262(R151)	5	2	0		1.4×10^8
ML4262(R931)	2	1	0		$>2.0 \times 10^9$

Mice were inoculated ip with 0.2 ml suspension prepared as in materials and methods. Deaths after 48 h are given. LD_{50} values were calculated by the Reed-Muench method using 10^9 bacteria per ml as the number present in an undiluted suspension.

Table 5. Properties of strains used in mouse protection tests

Strain	R factor markers	Gentamicin sensitivity	Other identifying properties
280	R^-	hypersensitive	brown pigment hypersensitive to many antibiotics
280(RUA7)	Cb Gm Sm Tc	sensitive	brown pigment rif^r
280(R130)	Gm Sm Su	moderately resistant 3N acetylating* enzyme	brown pigment rif^r
280(R151)	Cb Gm Sm Su	moderately resistant 2"-O-adenylylating* enzyme	brown pigment rif^r
PA-103	R^-	sensitive	pyocine type 10 exceptionally virulent
10804	R^-	moderately resistant permeability deficient	pyocine type 1h
1136	R^-	highly resistant permeability deficient	blue pigment pyocine type 16

Cb carbenicillin

Gm gentamicin

Sm streptomycin

Tc tetracycline

* position and type of modification of the gentamicin molecule

from hypersensitive to highly resistant. Strain 280 is a hypersensitive strain. Strain PA-103 and 280(RUA7) have MIC typical of sensitive strains. Resistant strains with either R factor directed enzymatically mediated resistance or permeability mediated resistance are included.

Other properties listed were used to confirm the identity of the strain. All are stable properties. Strains were periodically checked for presence of resistance markers and levels of gentamicin resistance. These properties remained stable throughout the course of the study.

Virulence Determinations

Choice of media. In preliminary tests, organisms were grown and prepared in NB. Using this media, 10-fold concentration of cultures at A_{600} of 0.5 was usually insufficient to kill 5 out of 5 mice. This media was abandoned when it was established that bacteria grown in BHI were at least 10 times more virulent.

Virulence in normal animals. Strains used for protection tests included 280, 280(R130), 280(R151) and 280(RUA7). Raw data of virulence tests and LD_{50} of these strains is given in Table 2 and Table 3. Three additional *P. aeruginosa* strains, PA-103, 10804, and 1136 were also used. Results of virulence tests for these three strains and those of 280 for comparison is given in Table 6. Strains 1136 and 10804 are similar to 280 in virulence. PA-103 is considerably more virulent. LD_{50} values in normal mice of all strains used are given in Table 8. Reproducibility of LD_{50} strains non-isogenic to 280 was comparable to that in 280.

Table 6. Virulence of non-isogenic *P. aeruginosa* strains for normal mice

Strain	Deaths per 5 mouse group				
	Concentration of suspension used as inoculum				
	x10	x1	$x10^{-1}$	$x10^{-2}$	$x10^{-3}$
280	5	4	0		
PA-103	5	4	4	2	1
10804	5	2	2	0	
1136	5	4	1	0	

Mice were inoculated ip with 0.2 ml of bacterial suspension prepared as described in materials and methods. Deaths after 48 h are given.

Induction of Neutropenia

Decrease in white blood cell count after a single ip injection of 300 mg/kg of cyclophosphamide is shown on Figure 1. The arithmetic mean of counts taken from four animals is shown as well as the range of counts.

White blood cell count drops steadily over the first four days to a low of about 20% of the original values. Counts for individual mice after four days ranges from 14 to 25% of those taken before treatment. Counts remained low for two days then began to rise again. The pattern observed agrees well with that reported by Pierson *et al.* (1976), from whom the method was taken.

No special precautions were taken in handling mice which received cyclophosphamide. No deaths were observed in cyclophosphamide treated mice not intentionally infected.

Infection of animals receiving cyclophosphamide were always carried out on day four after cyclophosphamide injection. As deaths were counted after 48 hours, this schedule allowed the completion of virulence and protection tests within the two days when white blood cell counts were at minimum.

Virulence tests in neutropenic mice. Mice made neutropenic with cyclophosphamide were used for virulence tests four days after cyclophosphamide treatment, at which time white blood cell counts were about 20 per cent of normal counts. Tests were done using the same procedure as used for normal mice.

Representative raw data are given in Table 7, and LD_{50} determinations are shown in Table 8. LD_{50} values for neutropenic mice were an

Figure 1. Effect of cyclophosphamide on mouse white blood cell count. Mice were given a single ip dose of 300 mg/kg cyclophosphamide on day 0. White blood cell counts were done at 24 hour intervals. Each point represents the arithmetic mean of counts obtained from four mice. Bars represent the range of counts.

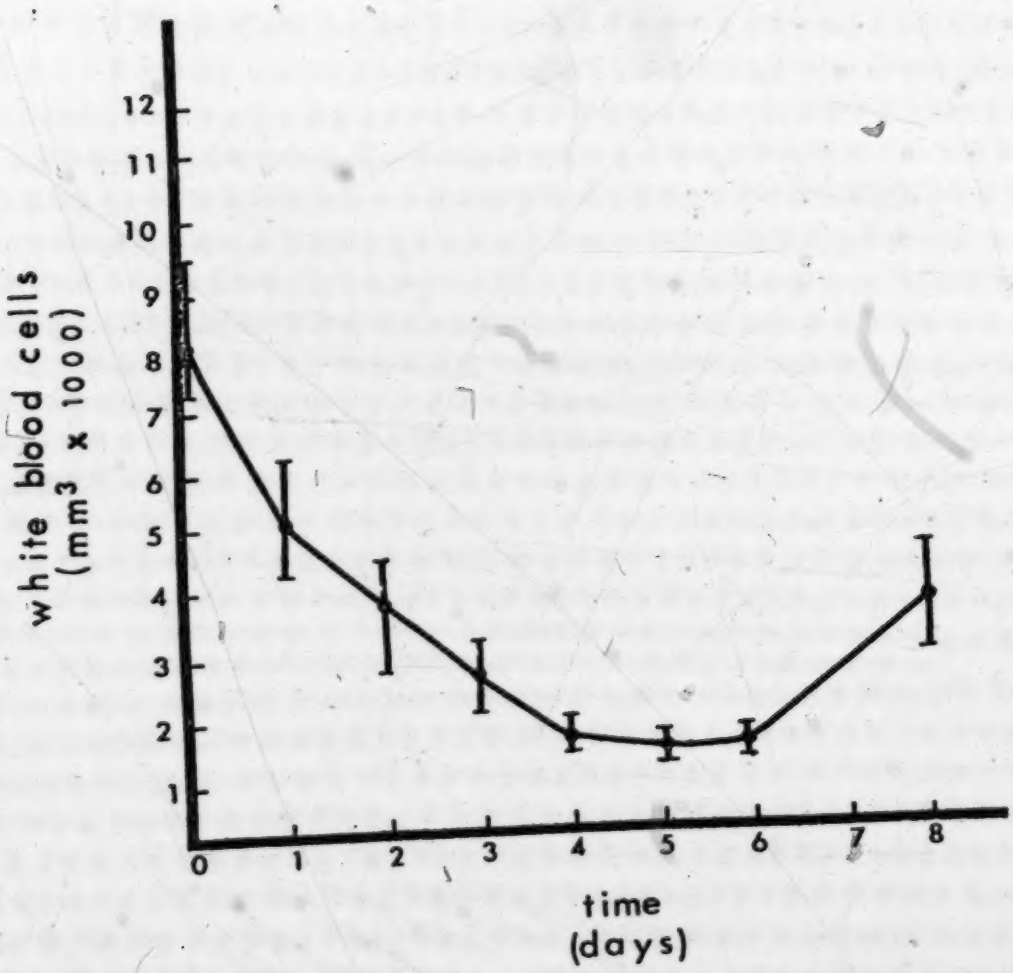

white blood cells
(mm³ x 1000)
12
11
10
9
8
7
6
5
4
3
2
1
0
1
2
3
4
5
6
7
8
time
(days)

Table 7. Virulence of *P. aeruginosa* for neutropenic mice

Strain	Deaths per 5 mouse group			
	Concentration of suspension used on inoculum			
	$x10^{-1}$	$x10^{-2}$	$x10^{-3}$	$x10^{-4}$
280	5	2	1	0
280(RUA7)	5	3	0	
280(R130)	5	3	0	0
280(R151)	5	3	2	
PA-103		5	5	0
10804	5	2	2	0
1136	5	3	0	

Tests were carried out by the same procedures as used for normal mice four days after cyclophosphamide treatment.

Table 8. LD_{50} of *P. aeruginosa* for normal and neutropenic mice

Strain	LD_{50} (bacteria/inoculum) Normal Mice	Neutropenic Mice
280	4.5×10^7	2.0×10^6
280(RUA7)	5.0×10^7	3.1×10^6
280(R130)	4.5×10^7	3.1×10^6
280(R151)	8.0×10^6	5.6×10^5
PA-103	1.6×10^6	6.3×10^4
10804	5.6×10^7	1.5×10^6
1136	4.8×10^7	3.7×10^6

LD_{50} were calculated by the Reed-Muench method using virulence data and plate counts.

average of 15.2 times lower than those for normal mice. LD_{50} values of individual strains ranged from 13 to 37 times lower. Reproducibility of LD_{50} values were somewhat lower in neutropenic mice than in normal mice. Variation was usually within a factor of $10^{0.4}$.

Because neutropenic animals are more susceptible to all infections, bacteria were recovered from heart tissue of dead animals and blood of moribund animals to establish that the strain administered was the cause of death. Strains 1136, 280 and 280 R^+ were identified by their characteristic pigment and antibiotic resistance patterns. Other strains were identified by pyocine typing. In all cases, it was possible to recover only the infecting strain.

Serum Levels of Gentamicin

Peak levels. Peak levels occurred within 10 minutes after sc injection. Gentamicin levels at 10 minutes are shown in Table 9.

As errors of ± one dilution commonly occur in the broth dilution method, levels obtained by this method may be considered only as rough estimates. These estimates were used to determine appropriate dilutions for the radioenzymatic assays. At least three samples at each level were assayed by the radioenzymatic method. Table 9 shows the mean level and standard deviation. Given the limitations of the broth dilution assay, levels determined by the two methods show reasonably close agreement.

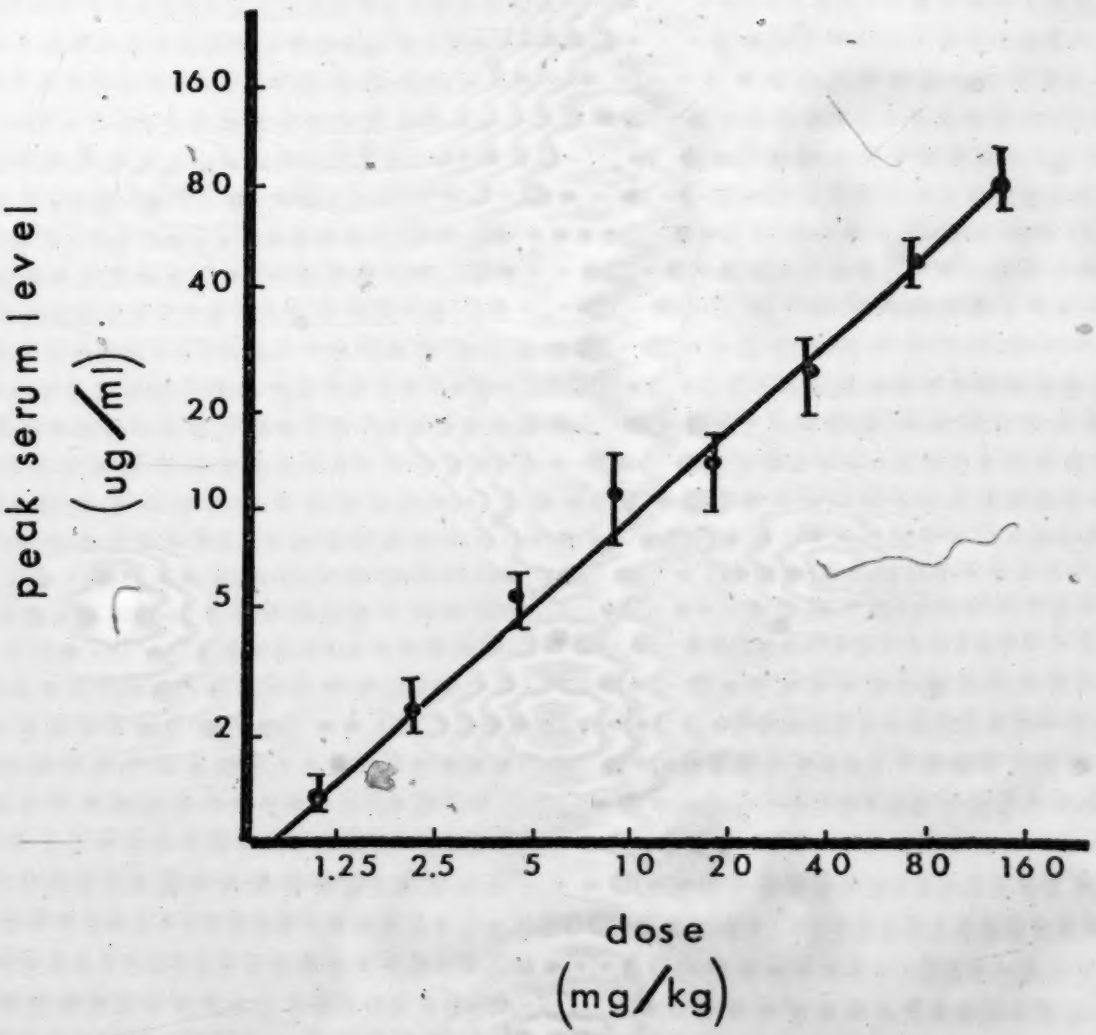
Peak levels obtained by the radioenzymatic method are plotted on Figure 2. A linear relationship between $\log_{10}$ dose and $\log_{10}$ peak serum level was obtained. Levels reported here are similar to those obtained by Waitz (1975) using a disc diffusion assay.

Table 9. Peak serum levels of gentamicin in mice measured by broth dilution and radio enzymatic methods after a single sc dose

Dose mg/kg	Serum level (µg/ml ± standard deviation) Broth dilution	Radioenzymatic
0.6	-	1.2 ± 0.2
1.25	1.3	1.4 ± 0.2
2.5	5	2.5 ± 1.2
5	5	5.7 ± 1.0
10	20	12.0 ± 4.1
20	20	14.0 ± 4.0
40	40	28.3 ± 7.5
80	80	75.0 ± 10.6
160	80	120.0 ± 21.2

Figure 2. Peak serum levels of gentamicin

Levels were determined for serum taken 10 minutes after a single sc dose and assayed by the radioenzymatic method. Points represent arithmetic mean values, bars standard deviations.


peak serum level
(ug/ml)
160
80
40
20
10
5
2
1.25
2.5
5
10
20
40
80
160
dose
(mg/kg)

Rates of clearance. Clearance of gentamicin from mouse serum is shown on Figure 3.

Each plot represents serum levels obtained from a single group of three or four mice. Assays were done by the radioenzymatic method. As this assay cannot be considered accurate below one µg/ml, assays at lower dosages were continued for one hour only.

Rates of clearance were fairly consistent in the range of dosage used. The time required for serum gentamicin level to drop by one half ($T_{1/2}$) ranged from 18 to 24 minutes (Table 10). No variation in rate of clearance with time was seen within the time course used. Rates of clearance observed were similar to those reported by Waitz (1975).

Levels after repeated doses of gentamicin. Subcutaneous doses were given every hour and serum collected 10 minutes after injection. One group of three mice was used at each dose level. Serum samples were assayed by the radioenzymatic method. Results are presented in Table 11.

A gradual buildup of gentamicin occurred over the course of administration. Total increase over six doses ranged from about 15 to 50 per cent.

Levels in neutropenic animals. Peak levels and clearance rates were determined in neutropenic animals at 5, 20 and 80 mg/kg doses. Levels were indistinguishable from those of normal animals.

Acute toxicity of gentamicin. No deaths were observed in mice given six hourly doses of gentamicin at 80 mg/kg. Eight doses at this level given at hourly intervals resulted in one death in ten mice. LD_{50} for six doses at hourly intervals calculated by the Reed-Muench method

Figure 3. Clearance of gentamicin

Each plot represents serum levels obtained from a single group of three or four mice. Assays were done by the radioenzymatic method.

2.5 mg/kg dose

5 mg/kg dose

10 mg/kg dose

20 mg/kg dose

40 mg/kg dose

80 mg/kg dose

Dotted line represents extrapolation from known points.

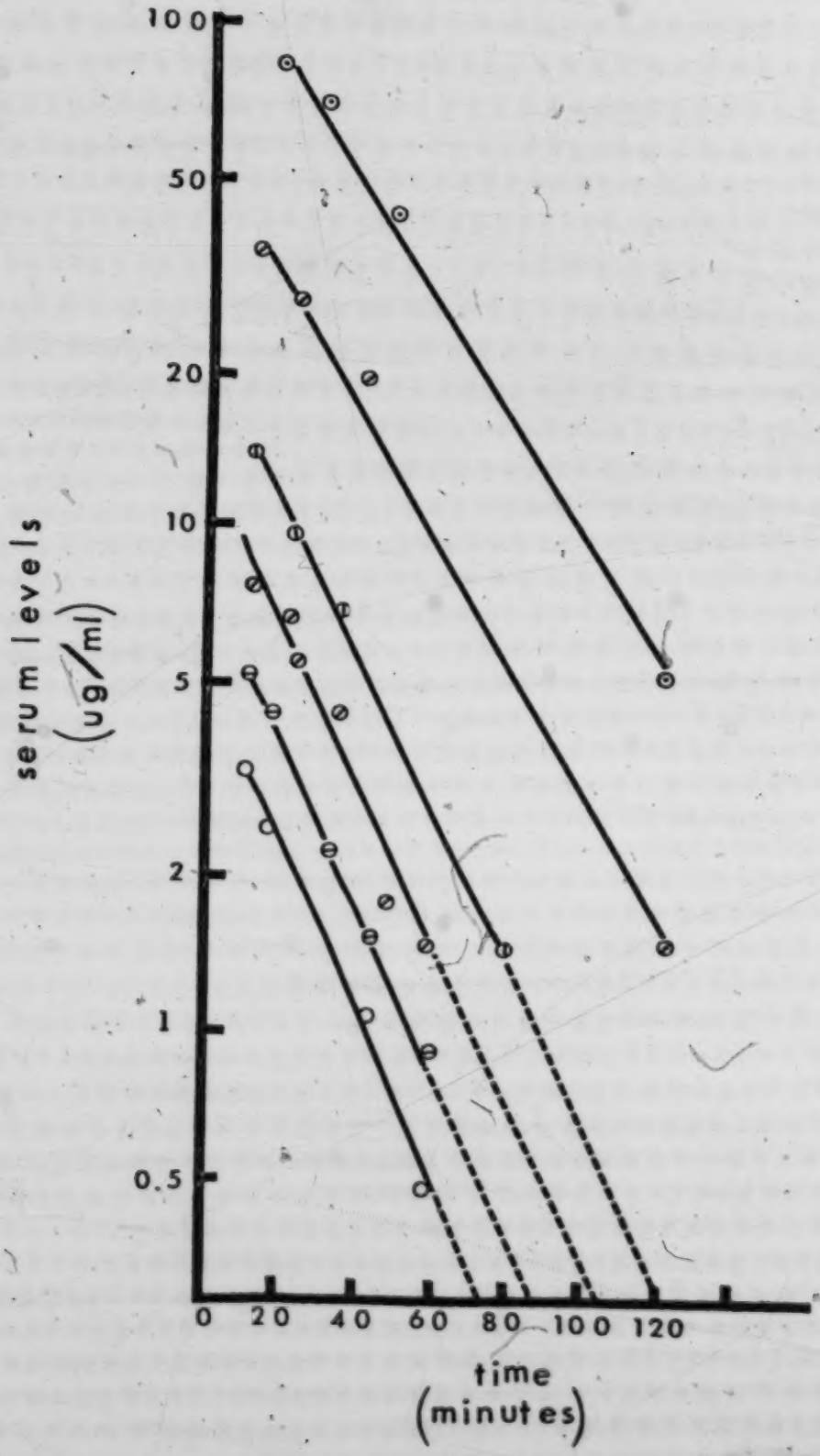

100
50
20
10
5
2
1
0.5
serum levels
(ug/ml)
0
20
40
60
80
100
120
time
(minutes)

Table 10. Rate of clearance of gentamicin after single sc dose

Dose (mg/kg)	$T_{1/2}$ (minutes)
2.5	18
5	20
10	20
20	22
40	24
80	24

Time required to reduce serum gentamicin concentration by one half ($T_{1/2}$) were calculated from Figure 3.

Table 11. Gentamicin levels after repeated doses

Dose (mg/kg)	Serum level (μg/ml)			
	Total doses given			
	1	2	3	6
2.5	3.5	3.8	3.9	4.3
5	5.0	5.5	6.1	7.0
10	8.0	9.0	9.8	12.7
20	15	17	19	23
40	35	40	49	61
80	83	92	101	140

Hourly sc gentamicin was given and serum sampled 10 minutes after dose. Serum was assayed by the radioenzymatic method.

was 178 mg/kg. Single doses of 160 mg/kg did not produce any deaths. The LD_{50} for a single dose was 210 mg/kg.

Eighty mg/kg for six doses and 160 mg/kg for single doses were the maximum levels used.

Protection Tests

Choice of dose schedule. The effectiveness of single dose and multiple dose schedules were compared in three strains. Results are summarized in Table 12. For two of the three strains six doses at hourly intervals produced no advantage over a single dose. For strain 280, however, the gentamicin PD_{50} obtained with six doses was significantly lower than obtained with a single dose. An eight dose schedule gave no further advantage. On the basis of these tests, a six dose schedule was chosen for all protection tests.

Choice of inoculum. Inoculum chosen was 50 times the LD_{50}. This dose was sufficient to kill five out of five control animals in about four out of five experiments. Control animals receiving 50 x LD_{50} and no antibiotic were used in all protection tests, and tests in which this dose was not sufficient to cause 100% mortality were discarded as invalid. To cover the possibility of too high a dose of infecting organisms being administered, a second control group was given 0.5 LD_{50} per mouse. If any mice in this group failed to survive, the test was discarded as invalid.

Protective levels of gentamicin. Representative raw data for protection tests done in normal animals are given in Table 13. The same tests done with neutropenic mice are shown in Table 14. In these tests

Table 12. Comparison of single versus multiple dose schedule for protection of mice against *P. aeruginosa* infection

Strain	PD_{50} (mg/kg gentamicin)		
	single dose	6 doses	8 doses
280	16	9	11
280(RUA7)	37	37	ND
PA-103	21	25	ND

ND not done

Mice given 50 x LD_{50} of bacterial suspension ip were given a single dose or multiple hourly doses (sc) of gentamicin commencing one hour after infection. Deaths were counted after 48 h and PD_{50} values calculated by the Spearman-Karber method.

Table 13. Protection by gentamicin of normal mice against *P. aeruginosa*

	Survivors per 5 mouse group						
	Dose (mg/kg gentamicin)						
Strain	2.5	5	10	20	40	80	160
280		0	3	5			
280(RUA7)			0	1	3	4	(5)
280(R130)			0	1	0	0	
280(R151)		(0)	1	0	3	5	
PA-103		0	1	2	3	5	
10804			0	0	1	4	(5)
1136			0	2	1	0	

Mice were given 50 x LD_{50} bacterial suspension (ip) and six hourly sc doses of gentamicin at the given levels. Survivors were counted after 48 h.

Bracketed figures are extrapolations used in calculation of PD_{50} values.

Table 14. Protection by gentamicin of neutropenic mice against *P. aeruginosa* infection

Strain	Survivors per 5 mouse group						
	Dose (mg/kg gentamicin)						
	2.5	5	10	20	40	80	160
280		0	1	5			
280(RUA7)	0	0	1	3	2	4	(5)
280(R130)		0	1	2	1	0	
280(R151)			0	1	4	5	
PA-103	0	2	0	4	5		
10804		0	0	2	2	3	(5)
1136			0	0	1	1	

Tests were done as for normal mice four days after cycophosphamide treatment.

mice were given 50 x LD_{50} of bacteria followed, beginning one hour later, with six hourly sc doses of gentamicin at the levels given. Survivors were counted after 48 h. Calculated protective levels are shown in Table 15. Protection tests were repeated at least twice. Calculated PD_{50} varied by a factor of $10^{0.5}$ or less.

No protection was possible for strains 280(R130) and 1136. Table 16 shows peak serum levels of gentamicin at the PD_{50} for those strains in which protection with gentamicin was possible. MIC determined in MHB are also shown. This table shows that the serum levels required to protect 50% of the mice from a given strain are considerably higher than the MIC of the strain--three to 36 times higher in normal mice and three to 28 times higher in the neutropenic mice.

In the three isogenic strains with similar virulence (280, 280(RUA7) and 280(R130)) the expected relationship between MIC and in vivo sensitivity is seen: less gentamicin is required to protect against 280 than against the less sensitive 280(RUA7), and no protection is possible against the relatively resistant 280(R130). However, it was possible to achieve protection against 280(R151) which has a resistance level similar to that of 280(R130). The level required was comparable to that required for 280(RUA7), which has an MIC four times lower than that of 280(R151).

It was not possible to achieve protection against strain 1136, as would be expected from its in vitro resistance. 10804, a moderately resistant or intermediate strain could be protected against only by very high levels of gentamicin. PA-103 was the most virulent strain in series, and consequently the one in which the lightest inoculum was used. Despite the light inoculum (especially in neutropenic mice), serum levels

Table 15. Gentamicin PD_{50} values for *P. aeruginosa* infection in normal mice

Strain	PD_{50} (mg/kg gentamicin)	
	Normal mice	Neutropenic mice
280	9 (7.9 - 10.2)	7 (6.3 - 7.6)
280(RUA7)	37 (27.9 - 50.6)	24 (16.2 - 26.9)
280(R130)	>80	>80
280(R151)	33 (26.9 - 46.0)	28 (22.9 - 34.7)
PA-103	23 (16.6 - 32.2)	12 (11.3 - 13.3)
10804	56 (48.0 - 66.8)	37 (25.6 - 53.7)
1136	>80	>80

PD_{50} values calculated by Spearman-Karber method. Bracketed figures are 95% confidence intervals by Spearman-Karber calculation.

Table 16. Comparison of MIC and serum levels of gentamicin required for PD_{50} in normal and neutropenic mice

Strain	MIC (μg/ml)	Serum level at PD_{50} dose (μg/ml) normal	neutropenic
280	0.25	9	7
280(RUA7)	2	30	24
280(R151)	8	27	23
PA-103	2	20	11
10804	4	44	30

MIC were done in MHB with Mg^{++} and Ca^{++} adjusted to physiological levels. Serum levels were calculated from Figure 2 (Peak serum levels of gentamicin) and PD_{50} values from Table 15.

of gentamicin at the PD_{50} are still much higher than the minimal inhibitory concentrations in vitro.

MIC after passage through animals. To ensure that the MIC of the bacteria was not changed by passage through animals, heart blood from moribund animals was collected, diluted 1:10 in MHB, grown to an A_{600} of 0.5 and used as an inoculum for MIC determinations. As the bacterial count of such blood was high ($\sim 10^6$ organisms/ml) this level was reached within two or three hours, minimizing effects of the media. MIC before and after passage are shown in Table 17. MIC are either the same or one dilution lower after passage.

MBC in presence of serum. To ensure that gentamicin had the same antibacterial effect in mouse serum as in MHB, minimal bactericidal levels were performed for strains 280 and PA-103 in MHB, equal parts MHB and serum, and serum alone. Bactericidal rather than inhibitory levels were determined because in the small volumes used it was difficult to visually discern an endpoint. MBC for both strains were identical in all three situations. Both were twofold higher than the MIC in MHB. MBC done in 50% pooled human serum and 50% MHB and MBC done in human serum alone had the same values as those done in mouse serum.

Inoculum effect in vivo and in vitro. Table 15 shows that PD_{50} values in neutropenic mice were somewhat lower than those in normal mice. As neutropenic animals were receiving at least 13 times fewer bacteria, the effect of inoculum sized on PD_{50} was examined in neutropenic animals. Table 18 shows PD_{50} of neutropenic mice given injections of 50 x LD_{50} and 500 x LD_{50}. Protection at the higher level was possible only for

Table 17. Comparison of MIC of *P. aeruginosa* before and after passage through mice

Strain	MIC (µg/ml gentamicin)	
	Before passage	After passage
280	0.25	0.25
280(RUA7)	2	1
280(R130)	4	4
280(R151)	8	8
PA-103	2	2
10804	4	2
1136	32	16

MIC were determined in MHB with 10^5 bacteria per ml inoculum.

Table 18. Effect of size of bacterial inoculum on gentamicin PD_{50} values for neutropenic mice

Strain	PD_{50}	
	Infection level	
	50 x LD_{50}	500 x LD_{50}
280	7	37
PA-103	12	.43
280(RUA7)	24	>80
280(R151)	28	>80

Protection tests were done as described in test. PD_{50} values were calculated by the Spearman-Karber method.

strains 280 and PA-103. This tenfold increase in inoculum size resulted in about a four to fivefold increase in PD_{50}. $\sim 500 \times LD_{50}$ for neutropenic animals is about half the dose given to normal animals in terms of total bacteria. Levels of gentamicin required for protection in neutropenic animals were about three to four times higher than those in normal animals given half as many bacteria.

In contrast to this substantial inoculum effect in vivo, Table 19 shows that a tenfold increase in bacterial inoculum in an in vitro testing system produces little effect on apparent sensitivity. MIC done with 10^5 and 10^6 bacteria per ml inocula are shown in Table 19. Most strains show no apparent change in resistance.

3. Minimal Inhibitory Concentrations of Gentamicin

MIC in six commercial media and minimal media with three different cation concentrations are shown in Tables 20 and 21. Mueller Hinton media were adjusted to physiological Mg^{++} and Ca^{++} concentrations.

NB, which is much lower in divalent cation content than the other complex media and which does not support growth to as high density gave much lower values than the other commercial media. MIC determinations done in NB had to be incubated for 48 rather than 16-24 hours to obtain reproducible endpoints.

MIC measured in the five other commercial media varied by no more than a single dilution factor within any strain. MHA gave the highest average MIC.

The effect of divalent cation concentration on apparent sensitivity is demonstrated by the MIC in minimal media. Increase of cation content in minimal media produced higher MIC. At physiological cation

Table 19. Effect of bacterial inoculum on MIC

Strain	MIC (µg/ml gentamicin)	
	Inoculum (bacteria/ml)	
	10^5	10^6
280	0.25	0.25
280(RUA7)	2	4
280(R151)	8	8
280(R130)	4	8
PA-103	2	2
10304	4	4
1136	64	64

MIC were done in MHB broth in one ml volumes.

Table 20. Minimal inhibitory concentration of gentamicin in commercial media

Strain	MIC (µg/ml gentamicin)					
	MHA (Ca 75) (Mg 20)	MHB (Ca 75) (Mg 20)	OxA (Ca 28) (Mg 4)	OxB	BHI	NB (Ca 9) (mg 4)
280	0.5	0.25	0.25	0.25	0.25	0.016
280(RUA7)	2	2	2	2	1	0.125
280(R130)	8	4	8	4	4	0.25
280(R151)	8	8	8	8	8	0.25
PA-103	2	2	1	2	2	0.125
10804	4	4	2	4	2	0.25
1136	64	64	32	16	32	1

Bracketed figures are concentrations in mg/l. Broth dilutions were done using one ml volumes in 16 x 125 mm tubes. Agar dilutions were done with a Steers replicator.

MHA Mueller-Hinton agar (BBL)

MHB Nueller-Hinton broth (BBL)

OxA Oxoid 'isosensitest' agar

OxB Oxoid 'isosensitest' broth

BHI Brain Heart infusion broth (BBL)

NB Nutrient Broth (BBL)

Table 21. Minimal inhibitory concentration of gentamicin in defined media

Strain	MIC (µg/ml gentamicin)		
	I (Ca 0) (Mg 4)	II (Ca 15) (Mg 4)	III (Ca 75) (Mg 20)
280	0.016	0.06	0.125
280(RUA7)	0.06	0.25	1
280(R130)	0.125	0.5	4
280(R151)	0.25	1	4
PA-103	0.06	0.25	1
10804	0.125	0.5	2
1136	2	4	16

Mice were done in one ml volumes in 16 x 125 mm tubes. Media varied only in calcium and magnesium concentration, which is given in mg/l in brackets.

concentration however, MIC in this minimal media were still less than those in complex media.

4. Uptake Studies

Preliminary studies showed that uptake of gentamicin by a given bacterial strain at lower concentration in NB than in other complex media with higher divalent cation content. Given the difficulties in obtaining and purifying [^{3}H] gentamicin, this property made NB the media of choice. It was also found in preliminary studies that uptake studies done in NB were more consistent and reproducible than those done in minimal media with comparable divalent cation content. NB was used for all uptake experiments.

Table 22 shows uptake of gentamicin in the presence of KCN compared to total uptake in the absence of KCN. In the presence of KCN, gentamicin accumulation in most cases shows only a small increase after 30 minutes relative to the initial uptake. For all strains, uptake in the presence of KCN beyond the initial instantaneous binding represents a very small proportion of the total uptake.

Uptake of gentamicin by sensitive and resistant strains is shown in Figure 4 and Figure 5. Initial uptake has been subtracted from total uptake in order to estimate energy dependent accumulation. The uptake curves showed excellent reproducibility under the conditions used.

Figure 4 shows uptake at 0.5 µg/ml of gentamicin. The three sensitive strains 280, PA-103, and 280(RUA7) show appreciable uptake. Both 1136, a highly resistant strain, and 10804, a moderately resistant strain, show negligible uptake. The two R factor carrying 280 derivatives, which are both moderately resistant, cannot easily be disting-

Table 22. Uptake by *P. aeruginosa* of gentamicin

Strain		Total uptake (ng gentamicin/mg dry bacteria)			
		Time (minutes)			
		0	10	20	30
280	KCN-	4.4	240	340	700
	KCN+	3.7	4.8	7.6	11.4
280(RUA7)	KCN-	3.0	49.9	115.0	176.2
	KCN+	3.6	5.6	6.6	10.2
280(R130)	KCN-	2.8	21.1	30.6	55.6
	KCN+	3.1	5.4	7.1	6.8
280(R151)	KCN-	5.0	18.2	54.1	97.3
	KCN+	3.0	5.1	5.4	4.8
PA-103	KCN-	3.5	59	202	319
	KCN+	3.7	6.0	5.5	6.6
10804	KCN-	3.6	7.6	5.6	5.5
	KCN+	5.2	5.4	7.4	5.3
1136	KCN-	5.3	3.6	4.5	6.6
	KCN+	4.3	11.3	5.2	3.1

Bacteria growing in mid log phase were exposed to 1 µg/ml tritiated gentamicin. 1.2 ml samples were collected on milipore filters which were then dried and counted. KCN to 1mM was added five minutes before the addition of gentamicin.

Figure 4. Energy-dependent uptake of gentamicin by *P. aeruginosa* at 0.5 µg gentamicin/ml.

Experiments were done in NB in shaking cultures at 37C. Energy dependent uptake was estimated by subtraction of instantaneous binding after addition of gentamicin.

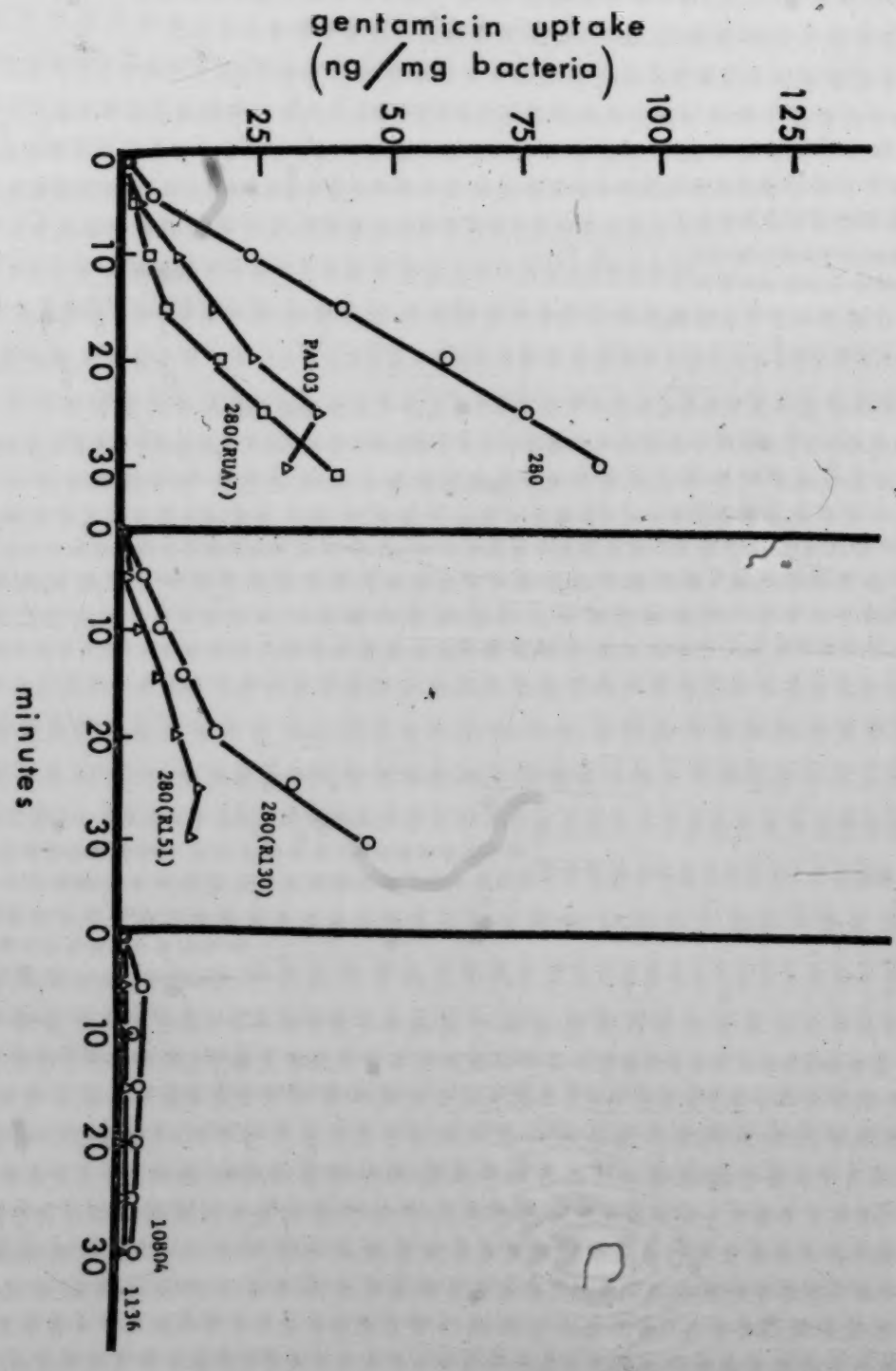
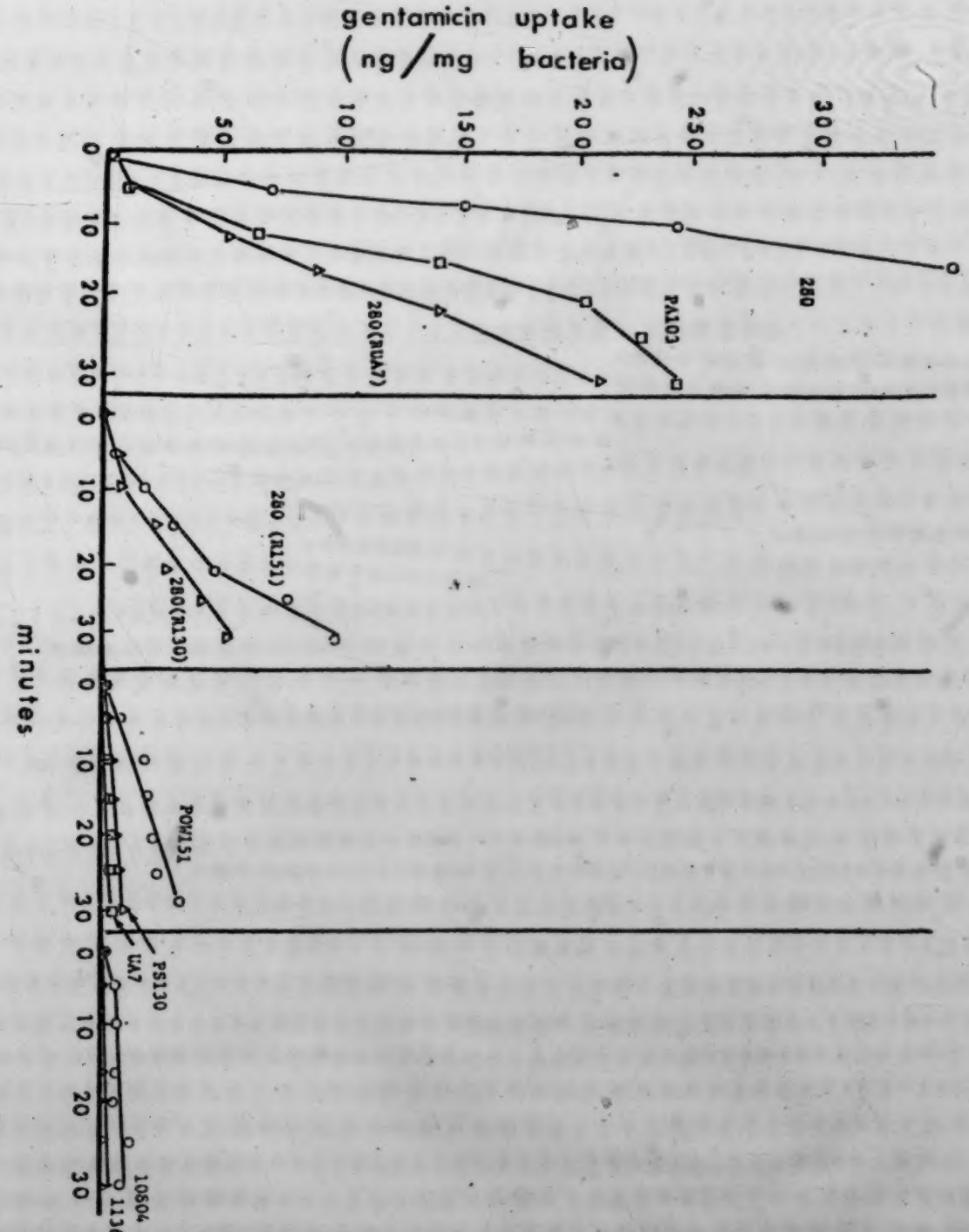

gentamicin uptake
(ng/mg bacteria)
25
50
75
100
125
0
10
20
30
PA103
280(RUA7)
280
280(RJ51)
280(RJ30)
10804
1134
minutes

Figure 5. Energy dependent uptake of gentamicin by *P. aeruginosa* at 1.0 µg gentamicin/ml.

Experiments were done in NB in shaking cultures at 37C. Energy dependent uptake was estimated by subtraction of instantaneous binding.


gentamicin uptake
(ng/mg bacteria)
50
100
150
200
250
300
0
10
20
30
280
PA103
280(RUA7)
280 (R151)
280(R130)
POM151
PS130
UA7
10804
1136
minutes

uished from the sensitive strains by their uptake of gentamicin at this level. In Figure 5, uptake of gentamicin at one µg/ml, differences between sensitive and resistant strains are magnified. The three sensitive strains take up relatively large quantities of gentamicin. The hypersensitive strain 280, accumulates more gentamicin than the other sensitive strains. As previously, the highly resistant strain 1136 shows negligible uptake, as does 10804 a moderately resistant "permeability deficient" strain. The two moderately resistant R factor-bearing strains take up more gentamicin than the permeability deficient strain, but are still easily distinguished from the sensitive strains.

Three naturally occuring strains with R factors were also used for uptake studies. These strains, PS-130, POW151 and UA7 were the sources of the R factors in the 280 derivatives used in this study. The three naturally occuring strains are all highly resistant in vitro. All showed low uptake.

By examination of the uptake curves it was decided that uptake after 25 minutes at one µg/ml gentamicin was the point on the curves at which sensitive and resistant strains were most clearly distinguishable. Twenty-two strains covering a broad range of sensitivity were examined for uptake at this point. Table 23 shows uptake after 25 minutes after subtraction of initial uptake. Uptake of less than 100 µg gentamicin/mg dry weight bacteria was chosen as a criterion for sensitive strains. All strains with MIC of eight or more accumulated less than 25 ng/mg, except for three R factor carrying derivatives of 280, which accumulated 33 to 76 ng/mg, so all would be classed as resistant. Typical sensitive strains took up 140 to 255 ng/mg while hypersensitive strains took up over 500 ng/mg. Among the six intermediate strains, five behaved as

Table 23. Gentamicin uptake by sensitive and resistant strains of *P. aeruginosa*

Strain	MIC (µg/ml)	Gentamicin uptake at 25 minutes (ng/mg dry weight bacteria)
280	.25	580
3503	.25	735
PA-103	2	220
280(RUA7)	2	140
917	2	142
900	2	242
9103	2	255
10804	4	10
931	4	125
SPI	4	24
11605	4	15
10055	4	11
11694	4	10
280(R130)	8	76
280(R151)	8	40
1136	64	1
280(R16)	16	33
13934	32	25
PS-130	64	10
POW-151	>64	23
UA7	>64	4
8803	>64	2

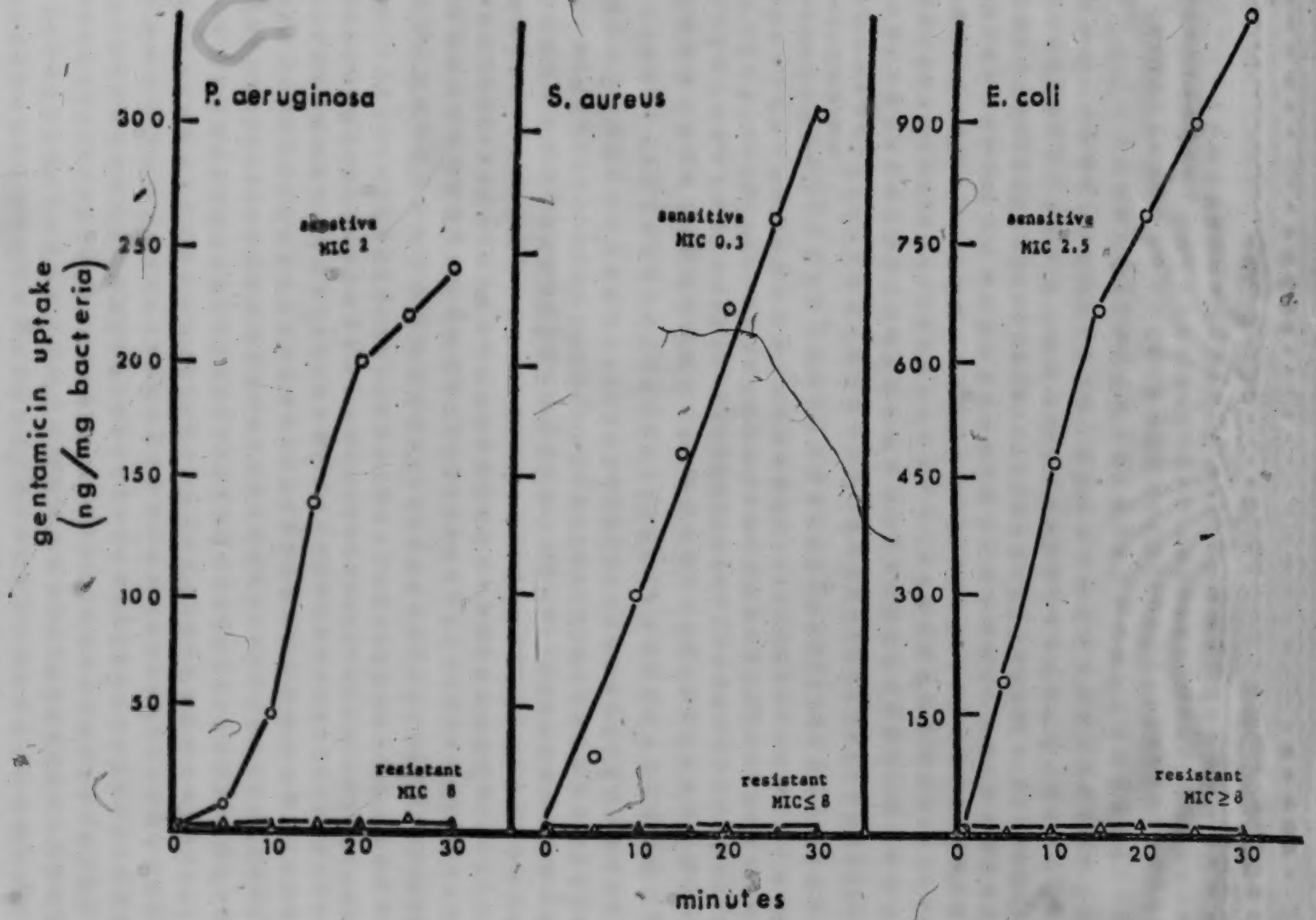
MIC were determined in MHB. Uptake was done in NB with 1 µg/ml gentamicin. Instantaneous binding of gentamicin was subtracted from total uptake in order to estaimate energy-dependent accumulation.

typical resistant strains with uptake of less than 25 ng/mg while one behaved as a typical sensitive strain with uptake of 120 ng/mg. Thus there was good correlation between sensitivity determined by MIC and accumulation.

Gentamicin accumulation in sensitive and resistant strains of *Staphylococcus aureus* and *Escherichia coli* was also examined. Results of uptake on these two strains and two representative *P. aeruginosa* strains are presented on Figure 6. Because *S. aureus* showed considerably more gentamicin binding in the presence of KCN than did the Gram negative strains, energy dependent gentamicin accumulation was determined by subtraction of accumulation of KCN controls rather than initial uptake. This was done for all points on Figure 6. As in the case of *P. aeruginosa*, sensitive and resistant strains of *S. aureus* and *E. coli* are clearly distinguished by uptake. Resistant strains of all three showed very little uptake. The sensitive *E. coli* used had an MIC similar to that of the sensitive *P. aeruginosa* but it accumulated much higher levels of gentamicin under our conditions. The sensitive *S. aureus* used took up quantities similar to the *Pseudomonas* strain despite its lower MIC.

Figure 6. Energy dependent uptake of gentamicin by sensitive and resistant bacteria.

Experiments were done in NB at 1 µg gentamicin per ml of media. Energy dependent uptake was estimated by subtraction of uptake in the presence of 1mM KCN. MIC done on MHA are given in units of µg/ml.


P. aeruginosa
S. aureus
E. coli
gentamicin uptake (ng/mg bacteria)
300
250
200
150
100
50
900
750
600
450
300
150
sensitive MIC 2
resistant MIC 8
sensitive MIC 0.3
resistant MIC≤ 8
sensitive MIC 2.5
resistant MIC≥ 8
0
10
20
30
minutes

DISCUSSION

The mouse protection test provides a relatively simple means of in vivo measurement of antibiotic activity. Some suggestions have been made for standardization of the test procedure, (Davis, 1975; Waitz, 1975) but at present differences in details of experimental design make comparison of results between laboratories difficult.

It is clear from our data that inoculum size is a major factor in determining PD_{50}. There is no agreement as to optimum bacterial inoculum for mouse protection tests. In several studies (Waitz *et al.* 1972; Weinstein, 1973; Weinstein *et al.*, 1971; Miller, 1976) a standard inoculum of 10^7 organisms was used. This is a relatively light challenge unlikely to be uniformly lethal other than with exceptionally virulent strains. Such studies have generally produced lower PD_{50} relative to MIC than we have shown. Given the wide variation of virulence possible in *P. aeruginosa* (Kobayashi, 1971; Davis, 1974), protective studies using such light inocula and no virulence controls are suspect. If uniformly lethal inocula are not used, the validity of the calculated PD_{50} is compromised. Other studies have adjusted inocula according to virulence, usually using 100 LD_{50} as the infecting challenge (Heifetz, 1972 and 1974; Davis, 1974; Meyers, 1976). Davis (1974) has stressed the need for virulence controls in all protection tests. The inoculum which we have used, 50 LD_{50} is near the minimum required to achieve a uniformly lethal infection. This is an obvious precondition for valid statistical analysis of lethality data. Use of such relatively high inocula however does present a very rigorous test of the antibiotic.

There is also no consensus as to the optimum dose schedule for

mouse protection tests with *P. aeruginosa*. Optimum timing appears to vary with the specific strain used, but some generalizations are possible: gentamicin given one hour after infection gives lower PD_{50} than gentamicin given one to one half hour before, and gentamicin given one hour after infection gives lower PD_{50} values than given after two hours or later (Waitz, 1975; Meyers, 1976). The increasing levels of antibiotic required with a delayed dose have been directly related to increase in the numbers of organisms (Waitz, 1975). Heifetz *et al.* (1974 and 1972) administered antibiotic concurrently with infection, but still found a wide gap between serum levels and MIC. Split dose schedules have been reported to show no advantage over single dose (Davis, 1975; Meyers, 1976).

We found that a multiple dose schedule of six hourly doses beginning one hour after infection was more effective than a single dose, and that two further doses gave no additional advantage. The six dose schedule we used for protection may mimic a human situation more closely than a single dose, as repeated doses may compensate for the relatively short $T_{1/2}$ of gentamicin in the mouse.

We found that there was no difference in bactericidal activity of gentamicin in mouse serum compared to human serum in in vitro MBC determinations. Thus the serum levels required for protection in mice may be relevant to human therapy.

LD_{50} values for normal mice for strains used in protection test and in comparison of R^- and R^+ strains were in the same range as those found in similar studies of virulence (Klynn and Gorrill, 1967; Kobayashi, 1971; Marolleau, 1977). Our LD_{50} values are typical of those found of *P. aeruginosa* isolated from either human or environmental sources.

Strain PA-103 has been previously reported as having a considerably higher virulence than we have found (Davis, 1975). However, lowered virulence for this strain has been shown by other workers as well (S.D. Davis, personal communication). This strain has been recommended as a reference strain for the standardization of mouse protection tests (Davis, 1975). However its variable virulence make it less suitable for this purpose than was hoped. This strain is known to produce a potent exotoxin (Liu, 1973; Davis, 1975). However this toxin does not account for virulence in mice (Davis, 1973; Kobayashi, 1971), and the reduction of virulence noted by us and by others is not related to toxigenic potential (S.D. Davis and P.-V. Liu, personal communications).

The magnitude of increase in susceptibility to pseudomonas infection after cyclophosphamide treatment is similar to that reported by others (Pierson *et al.*, 1976; Scott and Robson, 1977). It has been argued that studies of *P. aeruginosa* infection using normal animals cannot be clinically relevant because normal animals are not susceptible to *P. aeruginosa* and can be infected only by injection of very large numbers of organisms (Stieretz and Holder, 1975). Neutropenic animals may provide a more appropriate model. Cyclophosphamide-induced neutropenia produces an increase in susceptibility to pseudomonas infection which parallels that seen in burned animals (Stieretz and Holder, 1975) as well as that seen in hosts with neutropenia due to hematological malignancies or anticancer therapy.

The effect of R factors on virulence of *P. aeruginosa* has not been previously documented. Most of the R factors that we tested (six out of 11) did not affect virulence, while a smaller number (four out of 11) produced a significant decrease in virulence. One of the R factors

not affecting virulence and one which lowered virulence were found to have the same effect in two recipients, 280 and ML4262.

Loss of virulence following acceptance of R factors or other plasmids in *Salmonella typhimurium* has occasionally been documented (Smith, 1972; Krishnapalli and Karthigasu, 1972). In one case virulence could be regained without loss of R factor by repeated subculture (Thiele, 1970). No change in virulence after acquisition of R factors is an equally common finding (Smith, 1972; Watanabe, 1971).

The enhancement of virulence of 280 produced by (R151) is a novel finding. The virulence enhancing effect was stable after repeated subculture and apparent in both normal and neutropenic mice. This R factor did not produce a virulence enhancing effect in a second host. The basis for this virulence enhancing effect is unknown, and may provide a topic for further study.

The use of isogenic strains has allowed us to compare in vivo susceptibility of strains with different in vitro sensitivities without the complication of differences on virulence and inoculum size. 280 R^-. 280(RUA7) and 280(R130) are indistinguishable with respect to virulence. Only the hypersensitive strain, 280 R^-, had a PD_{50} at what is normally considered an acceptable serum gentamicin level. The MIC of 280(RUA7), 2 µg/ml of gentamicin, could be considered typical for a sensitive strain tested under rigorous conditions (Bryan and Van Den Elzen, 1977). However, the serum level required for protection is 37 µg/ml, a potentially toxic level. 280(R130) has a MIC of four to eight µg/ml, a level which is usually considered potentially susceptible, yet no protection against this strain could be shown at serum gentamicin levels up to 75 µg/ml. In this series it is clear that although in vitro sensitivity

testing may provide an indication of relative in vivo susceptibility, MIC levels are poor predictors of in vivo protective levels.

Protection was possible against 280(R151) which has a resistance level similar to that of 280(R130). As 280(R151) is considerably more virulent than the other 280 derivatives, a smaller inoculum was used for protection tests. The lowered level of antibiotic required to protect from 280(R151) relative to R(130) is a reflection of the importance of inoculum size on the effectiveness of the antibiotic in vivo. The somewhat lower PD_{50} values in neutropenic mice may also be partly due to an inoculum effect. Using neutropenic animals, we found that a 10-fold increase in inoculum raised the level of gentamicin required for protection by a factor of about four or five. This is similar to the inoculum effect observed in normal mice by Davis (1975). It would thus appear that the size of the bacterial inoculum is a major variable in determining the level of gentamicin required for protection. In contrast to this substantial inoculum effect in vivo, we were unable to show a significant inoculum effect in in vitro MIC, as is usually the case with aminoglycosides in vitro.

The major effect of the size of bacterial challenge on the required levels of gentamicin may have implications for human therapy. Best results in treatment of *P. aeruginosa* bacteremia are reported when antibiotic therapy is begun early or prophylactically (Tapper and Armstrong, 1974). These improved results may reflect the lower number of infecting organisms present early in infection.

In the three strains not isogenic to 280, we again found MIC to be inadequate as an indicator of therapeutic levels. It was not possible to protect against 1136, as would be predicted by its in vitro resistance.

10804 required very high gentamicin levels for protection.

PA-103 is of particular interest because as a highly virulent strain, bacterial inocula used in mice were relatively light, especially in neutropenic animals. Nonetheless, serum levels required for protection are much higher than could be predicted by their MIC--eleven times higher in normal mice, six times higher in neutropenic mice.

The gap between susceptibility in vitro and in vivo is lowest when MIC determinations are carried out in media which give relatively high MIC. Thus the MIC most likely to be in line with results of in vivo testing are those done with comparatively high calcium and magnesium content. Among the six commercial media we tested, BBL nutrient broth, which has an exceptionally low level of divalent cations, have the lowest, and hence least predictive, MIC. Mueller Hinton media with adjusted calcium and magnesium gave the highest MIC. The other commercial media gave MIC only slightly lower than the adjusted Mueller Hinton media. The importance of calcium and magnesium content of media is clearly illustrated by MIC done in minimal media with varying calcium and magnesium levels. Increased levels produced increased resistance. However, physiological levels of calcium and magnesium were not sufficient to raise levels to that of Mueller Hinton media, indicating that other factors besides divalent cation influence resistance. Among the factors that may be of importance in this particular medium are low phosphate level and low ionic strength. Bactericidal gentamicin levels in our adjusted MHB matched those done in mouse and human serum.

It is clear from our data that MIC are not adequate as predictors of in vivo susceptibility. We found that PD_{50} values in both normal and neutropenic mice required serum levels which grossly exceed MIC done by

conventional methods, even when a light bacterial challenge was used in the animals. This was still true when sensitivity tests were carried out in media adjusted to resemble physiological conditions.

This discrepancy cannot be attributed to antagonistic effects of serum, as we were able to show no difference in bactericidal levels of gentamicin in serum compared to adjusted Mueller Hinton media. Nor is altered antibiotic resistance after growth in animals likely to be a factor, since MIC done on freshly isolated bacteria without subculture showed that passage through animals did not alter in vitro resistance.

There are a variety of factors which may account for the discrepancy between gentamicin activity in vitro and in vivo. A major factor may be that concentrations of antibiotic in most tissues are generally appreciably lower than those of serum. For example, levels of gentamicin in bile, pleural pericardial and synovial fluid are one half to one fourth those of serum, and levels in cerebro-spinal fluid are still lower (Riff and Jackson, 1971). Tissue concentrations are likely to be of some importance in a widely disseminated infection such as the one used in our study. Aminoglycoside uptake (and therefore activity) is profoundly influenced by oxygenation, pH and ionic environment, all of which may vary in an animal system. The rate of bacterial metabolism is generally much lower in vivo (Smith and Pearce, 1974), and the metabolic state of the bacteria may also influence uptake. Binding of antibiotic to serum or tissue proteins is often invoked to explain lowered antibiotic activity in vivo, but in the case of gentamicin, it is generally agreed that serum protein binding is negligible under physiological conditions (Ramirez-Ronda, 1975; Riff and Jackson, 1971; Gorden *et al.*, 1972; Black *et al.*, 1963). In addition, in the in vitro system,

the bacteria are exposed to antibiotic for 18 to 24 hours, while in an animal system exposure to peak levels of antibiotic is of shorter duration. Thus it is not surprising that a discrepancy between in vitro and in vivo results is maintained even when in vitro systems are adjusted to match serum conditions. However, this does not diminish the need for the use of media with physiological levels of divalent cation.

Most of the factors affecting antibiotic activity in a human host should be active in a mammalian animal model. One example which we have shown is that serum bactericidal levels of gentamicin are the same in either mouse or human serum. The main advantages of the mouse protection test over other animal models are that relatively large numbers may be used and that it generates quantitative data which may be analysed to show protective levels with confidence limits. A major disadvantage of using a small animal in antibiotic studies is that the antibiotic is eliminated much more rapidly than it would be in a human host, thus greatly reducing the period of time in which bacteria are exposed to peak levels. We have attempted to compensate in part for this by giving repeated doses. However, we have found that repeated doses do not greatly improve efficacy over that of a single dose.

A second difficulty common to most animal models is the artificial nature of infection. It can be argued that the large number of organisms required to establish a uniformly lethal infection in normal animals provides too severe a test for the antibiotic. This view is supported to some extent by the in vivo inoculum effect which we have demonstrated. However, even when very low inocula were used, as in the case PA-103, there was a wide gap between serum levels at PD_{50} and MIC. Animals made neutropenic by cyclophosphamide may be more appropriate than

normal mice, as the resulting increase in susceptibility to pseudomonas parallels that seen in victims of burns or neutropenia from other causes. We found our conclusions regarding the discrepancy between in vivo and in vitro gentamicin efficacy to be the same whether it was based on data from normal mice or data from neutropenic mice.

Our model best parallels the situation of disseminated infection in a susceptible host. This is the situation in which the role of antibiotic is likely to be most critical because of the inadequacy of host defences. It is unlikely that our model is of much relevance to local infections such as eye or urinary tract infections. Caution is always required when attempting to apply the results of animal studies to a human situation. However, despite the reservations we have stated concerning our model, it should have at least as much relevance to human therapy as in vitro sensitivity testing.

Our major conclusion is that therapeutic levels of gentamicin are much higher than the inhibitory levels obtained from MIC determinations. Our results suggest better therapeutic results are likely when higher gentamicin levels are used. Alternatives to higher gentamicin doses include the use of more active aminoglycosides or when appropriate combinations of aminoglycosides and beta lactam antibiotics. Carbenicillin and gentamicin are known to act synergistically against some pseudomonas strains, and often produce improved therapeutic results. Tobryamycin is more active than gentamicin against *P. aeruginosa* (Neu, 1976), and new aminoglycosides with lower toxicity or higher activity are an area of active research.

Resistant strains are usually defined as those which have MIC above the maximum safe antibiotic level in man. This is generally

considered six to ten µg/ml in the case of gentamicin. Our results suggest that these standards for resistance are too high and likely to lead to an overestimation of susceptibility. Klatersky *et al.* (1974) has suggested that strains should be considered sensitive to antibiotic only if minimal bactericidal levels are four to eight times lower than achievable serum concentrations. Our results suggest that this is likely to be a more realistic outlook in practice. However if free cation content is adjusted to serum levels, MIC for gentamicin in *P. aeruginosa* are rarely below 2 µg/ml. If serum levels of four to eight times these levels are to be used, the margin between effective and toxic levels of gentamicin is virtually eliminated. Potentially toxic levels may be required for treatment of life threatening infections.

In our model MIC did have some predictive value in the sense that strains with low MIC were more likely to be successfully treated with lower doses. This loose correlation has occasionally been noted clinically (Jackson and Riff, 1974; Tapper and Armstrong, 1974). However, in our model this predictive value is not absolute, as the number of infecting organisms appeared to be an equally important variable in determining the PD_{50}. The implication to be drawn from the importance of numbers of organisms in determining antibiotic efficacy is that the earlier treatment is initiated the more likely it is to be successful.

In summary, the implications of our model for human therapy are that treatment is most likely to be successful when high levels of antibiotic are used and when antibiotic therapy is initiated early. MIC cannot be regarded as an accurate indication of serum levels required for treatment. This is true even when serum levels of cation are used in the media used for MIC determination, although such media provide a better

estimate of susceptibility than others.

We have attempted to use the current knowledge of the relationship between gentamicin accumulation and gentamicin susceptibility to develop a practical, rapid method of distinguishing sensitive and resistant strains of *P. aeruginosa*. The principal advantage of this method is that it can produce susceptibility results from a growing culture in as little as one hour compared to 18 to 24 h by growth inhibition techniques. Its major disadvantage is the expense involved in the use of radioisotopes. We chose NB as the most suitable media for uptake because of the relatively low levels of gentamicin at which uptake began. The high cost and lengthly purification of tritiated gentamicin make this an important property. We found that the reproducibility of uptake curves in NB was very good, much better than in the one minimal media we tried.

Gentamicin accumulation includes an early energy independent binding to the cell envelope. This phase is not associated with loss of viability. It is the only phase which occurs in the presence of respiratory inhibitors such as KCN (Bryan and Van Den Elzen, 1975). We found that uptake over 30 minutes in the presence of KCN was not significantly greater than the initial instantaneous binding for *P. aeruginosa*. This allowed us to use gentamicin accumulation at zero time in the uptake assay as an estimate of energy independent background binding. By subtracting this value from the total elimination we could measure energy dependent binding without use of a cyanide poisoned control.

We found that at one µg gentamicin/ml sensitive and resistant strains could be easily distinguished by their uptake pattern. At a lower level, two R factor carrying strains of intermediate resistance

were not distinguishable from sensitive strains. At one µg gentamicin/ml these two strains still take up more gentamicin than permeability deficient strains with similar MIC, but could nonetheless be distinguished from sensitive strains. Sensitive strains accumulated higher levels of gentamicin, with the hypersensitive strain showing highest uptake. The three 280 derivatives we used in the uptake studies are laboratory artifacts. The situation of an R factor in a hypersensitive background is unlikely to exist in nature. The naturally occuring strains that were the source of these R factors were also exaimined. They showed very low uptake more similar to the permeability deficient strains 1136 and 10804.

Based on the uptake curves of representative strains we chose uptake after 25 minutes at one µg gentamicin per ml of NB as a point at which sensitive and resistant strains could best be differentiated and accumulation of more than 100 ng gentamicin per mg bacteria at this time as a lower limit for classification as a susceptible strain. Using this protocol we examined 22 strains of known sensitivity. In this system all strains with an MIC of two or less would be considered susceptible. All strains with an MIC of eight or more appear resistant. Among the six strains with intermediate MIC, five behaved as resistant strains showing very little uptake, and one behaved as a sensitive strain with uptake of more than 100 µg/ml. Given the inherent inaccuracy of the MIC determinations, this is adequate agreement. This method for determining uptake which we used does not discriminate between moderately resistant strains (MIC four to eight) and highly resistant strains. Both appear to be resistant. However, we believe that our own in vivo results and the accumulated evidence from previous study suggest that the possibility of

therapeutic success with such "moderately resistant" or "intermediate" strains is small, and that such strains are best classed as resistant. The cutoff point which we chose is well above the uptake levels of typical resistant strains so that any error in application of the technique would lead to overestimation of resistance, rather than the more serious error of overestimation of susceptibility. Our results with sensitive and resistant *S. aureus* and *E. coli* suggest that it will also be possible to determine susceptibility to gentamicin by accumulation studies in these strains.

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